IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re application of: **David B. Weiner**

Confirmation No: **2356**

Serial No: **10/734,024** Group Art Unit: **1648**

Filed: **December 11, 2003** Examiner: **Louise Wang Zhiying Humphrey**

For: **COMPOSITIONS AND METHODS FOR THE ABROGATION OF CELLULAR PROLIFERATION UTILIZING THE HUMAN IMMUNODEFICIENCY VIRUS VPR PROTEIN**

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPELLANTS' APPEAL BRIEF PURSUANT TO 37 CFR §41.37

Appellants hereby submit one copy of the present Appeal Brief to the Board of Patent Appeals and Interferences ("the Board") in response to the Final Rejection, dated November 19, 2008, in connection with the above-identified application. A Notice of Appeal was timely filed March 19, 2009. The period for filing the Appeal Brief has been extended, by enclosure of a petition and payment of the appropriate fee, to and through October 19, 2009.

Appellants respectfully submit that this brief complies with 37 C.F.R. § 41.37. This brief contains the items required by 37 C.F.R. § 41.37, under appropriate headings, and an authorization to charge the fee set forth in 37 C.F.R. § 41.20(b)(2).

(i) Real Party in Interest

The real party in interest in the above-identified patent application is the assignee, the Trustees of the University of Pennsylvania of Philadelphia, Pennsylvania. The real party in interest is referred to herein as "Appellants."

(ii) Related Appeals And Interferences

A Notice of Appeal in connection with United States Patent Application Serial No. 10/734,024, was filed April 30, 2007, and a corresponding Appeal Brief was filed November 30, 2007. The Appeal was not heard by the Board, but, instead, the Office withdrew the pending rejections of the claims and reopened prosecution on February 20, 2008. A copy of the Non-Final Office Action withdrawing the pending rejections is included in the Related Proceedings Appendix.

(iii) Status Of Claims

The present application was originally filed December 11, 2003, with claims 1-13.

A Preliminary Amendment and Response to Restriction Requirement was filed January 30, 2006 in which claims 6-13 were canceled and new claims 14-28 were added, whereupon claims 1-5 and 14-28 were pending.

A Response and Amendment was filed August 16, 2006 in response to a Non-Final Rejection of the claims, in which claims 19, 24 and 28 were canceled and new claims 29-34 were added, whereupon claims 1-5, 14-18, 20-23, 25-27 and 29-34 were pending.

A Response and Amendment was filed April 30, 2007 in response to a Final Rejection of the claims, requesting that claims 1-5, 14-18, 20, 25-27 and 29-31 be canceled.

An Advisory Action dated June 13, 2007 indicated in Item 7 that for purposes of appeal, the proposed amendment would be entered, whereupon claims 21-23 and 32-34 were pending.

A Notice of Appeal was filed April 30, 2007, and a corresponding Appeal Brief was filed November 30, 2007, whereupon claims 21-23 and 32-34 were pending.

A Response and Amendment was filed August 19, 2008, in response to a Non-Final Rejection of the claims, requesting that claim 21 be amended, whereupon claims 21-23 and 32-34 were pending.

Accordingly, claims 21-23 and 32-34 are pending and rejected, and claims 1-20 and 24-31 are canceled. The rejection of claims 21-23 and 32-34 is being appealed.

Claims 21-23 and 32-34, which are at issue in this appeal, appear in the Claims Appendix.

(iv) Status Of Amendments

A Response and Amendment was filed August 19, 2008, in response to a Non-Final Rejection of the claims, requesting that claim 21 be amended, whereupon claims 21-23 and 32-34 were pending.

No claims have been amended subsequent to the Final Rejection mailed November 19, 2008.

(v) Summary of Claimed Subject Matter

There are two independent claims pending in the present application: claims 21 and 32. Claims 22 and 23 are dependent on claim 21, and claims 33 and 34 are dependent on claim 32.

Claim 21 relates to methods of preventing lymphocyte activation. The method comprises the steps of obtaining isolated Vpr protein and contacting lymphocytes with an amount of the Vpr protein effective to prevent activation. Support for claim 21 can be found in claim 4 as originally filed and in the specification, such as, for example, at page 7, lines 16-26; page 9, lines 11-14, page 10, lines 16-20 and 27-34, and page 11, lines 3-8.

Claim 22 is dependent on claim 21 and relates to methods of claim 21 in which the lymphocytes are T cells.

Claim 23 is dependent on claim 21 and relates to methods of claim 21 in which the lymphocytes are B cells.

Claim 32 relates to methods of inhibiting lymphocyte activation. The method comprises the steps of obtaining isolated Vpr protein and contacting lymphocytes with an amount of the Vpr protein effective to inhibit activation wherein cytokine production and secretion that occurs due to lymphocyte activation by immunoglobulin is inhibited. Support for claim 32 can be found in the specification, such as, for example, at page 10, lines 14-20.

Claim 33 is dependent on claim 32 and relates to methods of claim 32 in which the lymphocytes are T cells.

Claim 34 is dependent on claim 32 and relates to methods of claim 32 in which the lymphocytes are B cells.

(vi) Grounds of Rejection to be Reviewed on Appeal

The first ground of rejection to be reviewed on appeal is whether claims 21-23 are unpatentable under 35 U.S.C. § 112, first paragraph as allegedly failing the enablement requirement.

The second ground of rejection to be reviewed on appeal is whether claims 32-34 are unpatentable under 35 U.S.C. § 112, first paragraph as allegedly failing the enablement requirement.

(vii) Argument

A. Claims 21-23

The rejection of claims 21-23 as being unpatentable under 35 U.S.C. § 112, first paragraph, was erroneous because the Office failed to set forth reasoning or evidence to support the rejection and cast any objective doubt on the truth of Applicant's assertion that the claimed invention is enabled. The Office failed to meet its burden to establish with reasoning and evidence that one skilled in the art would require undue experimentation to practice the claimed invention. Appellants respectfully urge that the rejection was therefore improper and should be reversed.

The Office asserts that Appellants have not provided any teaching regarding the prevention and inhibition of lymphocyte activation (Office Action, Nov. 19, 2008). Appellants expressly disclose the prevention and inhibition of lymphocyte activation in the specification including a description of production and secretion of cytokines by T cells, B cells, and monocytes. The reasons provided in support of the rejection was the absence of any supporting evidence to demonstrate the operability of the invention.. The only evidence cited was the reference Rogel, et al. which disclosed that Vpr protein is biological active and , when contacted with Vpr, cells undergo cell cycle arrest and discontinue passing through the cell cycle. Rogel et al. is completely silent with respect to both activation of lymphocytes. The rejection is an unsupported allegation that the claims are not enabled because Appellants have not shown them to be so.

It is well established that in the examination of claims for compliance with the enablement requirement, the Office bears the initial burden to establish non-enablement. That is, the object truth of an applicants' assertion that the claims are enabled is to be accepted unless and until the Office can put forward the arguments, supported by sound reasoning and evidence, as to why one skilled in the art would doubt such assertion. In order to make a rejection, the examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. *In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993) (examiner must provide a reasonable explanation as to why the scope of

protection provided by a claim is not adequately enabled by the disclosure). A specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as being in compliance with the enablement requirement of 35 U.S.C. 112, first paragraph, unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. Assuming that sufficient reason for such doubt exists, a rejection for failure to teach how to make and/or use will be proper on that basis. *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971). As stated by the court, "it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own ***with acceptable evidence or reasoning which is inconsistent with the contested statement***. Otherwise, there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure." 439 F.2d at 224, 169 USPQ at 370 (emphasis added). The law regarding enablement fully supports Appellants' position. The law is clear that applicants do not have to submit proof of operability to comply with the enablement requirement, and is equally clear that the absence of such proof alone is insufficient to meet the Office's burden.

Claims 21-23 refer to methods of preventing lymphocyte activation. The steps set forth in independent claim 21 include the step of "obtaining isolated Vpr protein" and the step of

> contacting lymphocyte cells with an amount of said Vpr protein effective to prevent activation.

In describing the methods, the specification clearly discloses that Vpr prevents lymphocyte activation. For example, on page 9 lines 11-14 of the specification states:

> It has been discovered that HIV protein vpr inhibits cell proliferation, induces undifferentiated cells to differentiate, that vpr effects modifies the state of macrophage cells, and prevents activation of lymphocytes.

On page 10, lines 14-20, the specification clearly describes what happens when activation is inhibited or prevented, stating

> It has been discovered that activation of lymphocytes such as T cells B cells and monocytes can be inhibited by vpr. Vpr prevents activation of these cells by immunoglobulin molecules. Activation of these cells by immunoglobulin molecules results in cytokine production/secretion. Accordingly, vpr inhibits cytokine production/secretion by these cells due to immunoglobulin activation.

In the Official Action dated February 20, 2008, the claims were rejected under 35 U.S.C. 112, first paragraph, for failing the enablement requirement. The Office incorrectly characterized the content of the specification as providing "no guidance regarding practice of the claimed methods" (page 5). On page 5, the Office erroneously concludes that the mechanism of action of Vpr in cells which is disclosed in the specification - that is the interaction of Vpr and the cellular protein Rip-1 which interacts with the glucocorticoid reception complex - is "not remotely related to inhibition or prevention of lymphocyte activation." The Office did not cite a single reference that discusses activation of lymphocytes and the involvement of the glucocorticoid receptor complexes. The Office, instead, cited three references to suggest that the prior art is "unpredictable" by stating the following:

> [a]t the time of the invention was made, successful implementation of lymphocyte activation inhibition [sic] and prevention with Vpr was not routinely practiced by those of ordinary skill in the art. Prior art only teaches T lymphocytes to secrete [sic] cytokines upon activation (Mosmann, 1997) and B lymphocytes to produce [sic] immunoglobulins once activated by cytokines (Paul, 1987). The only effect of Vpr expression within cells is the alteration of distribution of cells in the cell cycle and thereby mediating [sic] the prevention of cell proliferation (Rogel, February 1995)

Nothing in these three references establish any unpredictability with respect to the claimed invention. The specification is in agreement with the teachings of Mosmann, 1997 which describe the association of secretion of cytokines following activation of T cells. Likewise, nothing in Paul, 1987 contradicts the assertion that Vpr can prevent B lymphocyte activation. Finally, Rogel, February 1995 discloses that Vpr has biological activity although their observation were limited to linking Vpr expression with preventing cell proliferation; they did not study the effect of Vpr on lymphocyte activation. Accordingly, the reference did not make a reasoned argument why Appellants assertions of enablement should be questioned and the evidence they cited in support of the rejection did not support any assertion of unpredictability.

In Appellants' response filed August 19, 2008, Appellants pointed out that the Office had failed to establish a prima facie case of enablement. Having no obligation to do so, Appellants pointed out the relevant passages of the specification that describe lymphocyte activation and glucocorticoid receptor complex function disclosed above. Such response should have been sufficient to have the rejection withdrawn or provided anew with reasoning and support sufficient to meet the Office's burden.

Instead, in the Official Action dated November 19, 2008, the claims were rejected under 35 U.S.C. 112, first paragraph, for failing the enablement requirement. The Office restated the erroneous conclusion provided in the earlier Official Action, stating on page 4 that:

> [t]he disclosure fails to provide any guidance pertaining to the structural characteristics or mechanism of the interaction between Vpr and lymphocytes. The specification specifically discloses in more details and in working examples the use of Vpr or Rip-1-binding fragments of Vpr protein as transfection agent [sic] for the delivery of conjugated nucleic acid molecule [sic] or derivatives into the nucleus of a cell (page 36-37), which is not remotely related to inhibition or prevention of lymphocyte activation in vitro or in vivo, especially inside humans.

The rejection as set forth in the November 19, 2008 is improper in that it does not provide the requisite reasoning and evidence to support an initial conclusion of non-enablement. Rather, the Office focuses on a description of certain activities of Vpr directed at other claims not pending. The passages noted in the Official Action in no way contradict the claimed invention but rather are simply directed to other subject matter. The inclusion in the specification of a detailed description of a mechanism of action of another feature of Vpr does not undermine the assertion of enablement. More importantly, there is no requirement to provide a description of the mechanism by which the claimed invention works.

In Appellants' response filed August 19, 2008, Appellants pointed out the following on page 5:

> The Office has failed to set forth any reasoning or evidence to support the rejection. The Office has not established that the claimed invention does not meet the enablement requirement. Failing to do so, the burden is not properly shifted to Applicants. Applicants respectfully urge that the evidence and reasoning in the specification supports the conclusion that one skilled in the art would accept Applicant's assertion that the claims are enabled by the specification. In the absence of any evidence and reasoning in support of the rejection, Applicants are not required to put forth any evidence.
>
> Applicants respectfully request that any rejection based upon the Office statement above be withdrawn as the statements are not an accurate representation of the content of the specification. The Office has failed to provide any objective evidence or reasoning to question the validity of the contents of the specification. The Office has failed to present a single cited reference that demonstrates the claimed invention would not work. The Office has, instead, chosen to assert conclusory remarks about what one of ordinary skill in the art would not know based upon a negative inference drawn from references that do not discuss Vpr activity. The

Office reasoning is therefore flawed and the evidence provided by the Office is not relevant.

The claims are fully enabled. There is no reasoning provided by the Office or evidence of record that suggests the Appellants assertions are in objective doubt. A simple, unsupported conclusion does not negate the Appellants position. A simple conclusion unsupported by any objective reasoning n of evidence is insufficient to establish a prima facie case of non-enablement. The Office has failed to fulfill their burden of proving that the specification does not enable the claimed invention.

Appellants vigorously urge that the rejection should be reversed because the Office has failed to meet its burden. The references cited by the Office do not establish the unpredictability in the field. On the contrary, they are wholly consistent with the enablement of the invention. The absence of working examples and the absence of a disclosure of the molecular mechanisms involved together with the cited references simply does not establish that the claimed invention is not enabled. Accordingly, the burden has not shifted to Appellants and the assertions of enablement should remain accepted.

Although unnecessary to teach how to make and use the claimed invention, the specification does indeed describe a mechanism of action involving Vpr which provides a consistent explanation for the prevention of lymphocyte activation by Vpr. The glucocorticoid receptor complex is commonly known to inhibit the function of cytokine transcription. The specification clearly and expressly teaches that HIV Vpr interacts with Rip-1, a protein component of the glucocorticoid receptor complex. The specification specifically discloses that Rip-1 is found in lymphocytes (Specification, page 46, lines 25-37) and that Vpr interacts with Rip-1 (Specification, page 49, lines 1-21). The specification specifically discloses that steroids induce the translocation of Rip-1 across the nuclear membrane. (Specification, page 48, lines 31-37) while glucocorticoid receptor antagonists prevent such translocation. The specification notes that steroids are known to have immunosuppressive effects. The specification specifically discloses that Vpr binds to Rip-1 which is part of the glucocorticoid receptor complex and

prevents translocation of the complex. Accordingly, the specification discloses that Vpr and steroids have a similar function of translocating the glucocorticoid receptor to the nucleus. The specification discloses that one of Vpr's functions is its function as a translocation factor for glucocorticoid receptor; steroids are also translocation factor for glucocorticoid receptor. Steroids are immunosuppressants and the translocation of the glucocorticoid receptor to the nucleus is linked to this activity.

The claimed invention involves the step of contacting lymphocytes with an amount of Vpr protein effective to prevent lymphocyte activation. The specification plainly and expressly discloses the well-known biological function of glucocorticoids and their effects on the immune system. The specification demonstrates that Vpr mimics the biological function of dexamethasone and hydrocortisone, well-known glucocorticoids. Thus, one skilled in the art would expect that Vpr, which has a function that is like the nuclear translocation function of steroids, would be immunosuppressive. Moreover, when all of the evidence of record is considered, one skilled in the art would conclude that there is no reason to doubt Appellants' assertions of enablement.

While acknowledging that there is no requirement under the law for a patent application to contain a working example, the Office has asserted that a working example is necessary in the instant case because the Office the Rogel et al. reference discloses that Vpr functions to inhibit cell proliferation. The fact that a reference describes a function of Vpr other than that in the present invention is insufficient to establish that the state of the art and invention are unpredictable.

This reasoning is flawed. One skilled in the art would not consider the fact that Vpr can inhibit cell proliferation as somehow making an assertion that Vpr prevents lymphocyte activation unpredictable. The two functions are not contradictory. The Rogel reference is does not establish, as the Office incorrectly states, that the "prior art is unpredictable". Likewise the Paul et al. reference is not contradictory with a finding of enablement. Moreover, the Mosmann reference actually supports a finding of enablement. There is no evidence provided by the Office that contradicts the plain and express statements of the specification that Vpr prevents

lymphocyte activation. There is no evidence of record that contradicts the Appellants' disclosure of contacting lymphocyte cells with an amount of Vpr effective to prevent lymphocyte activation or raises any doubts as to why Appellants' assertions should be questioned.

Having submitted no reasoning or evidence that would cast objective doubt on the truth of the Appellants' statements in the specification, the Office has failed to sufficiently satisfy its burden to demonstrate that the claims are not enabled. When viewing the evidence of record in total, the evidence highly weighs in favor of finding the claims sufficiently enabled by the specification.

One skilled in the art would not conclude that the Office has met its evidentiary burden with respect to the enablement rejection. The rejection of claims 21-23 as failing the enablement requirement is erroneous. For the forgoing reasons, Appellants respectfully request that the rejection of claims 21-23 as being unpatentable under 35 U.S.C. § 112, first paragraph, as allegedly failing the enablement requirement, be reversed.

B. Claims 32-34

The rejection of claims 32-34 as being unpatentable under 35 U.S.C. § 112, first paragraph, was erroneous because the Office failed to set forth reasoning or evidence to support the rejection and cast any objective doubt on the truth of Applicant's assertion that the claimed invention is enabled. The Office failed to meet its burden to establish with reasoning and evidence that one skilled in the art would require undue experimentation to practice the claimed invention. Appellants respectfully urge that the rejection was therefore improper and should be reversed.

The Office asserts that Appellants have not provided any teaching regarding the prevention and inhibition of lymphocyte activation (Office Action, Nov. 19, 2008). Appellants expressly disclose the prevention and inhibition of lymphocyte activation in the specification including a description of production and secretion of cytokines by T cells, B cells, and monocytes. The reasons provided in support of the rejection was the absence of any supporting evidence to demonstrate the operability of the invention.. The only evidence cited was the

reference Rogel, et al. which disclosed that Vpr protein is biological active and , when contacted with Vpr, cells undergo cell cycle arrest and discontinue passing through the cell cycle. Rogel et al. is completely silent with respect to both activation of lymphocytes. The rejection is an unsupported allegation that the claims are not enabled because Appellants have not shown them to be so.

It is well established that in the examination of claims for compliance with the enablement requirement, the Office bears the initial burden to establish non-enablement. That is, the object truth of an applicants' assertion that the claims are enabled is to be accepted unless and until the Office can put forward the arguments, supported by sound reasoning and evidence, as to why one skilled in the art would doubt such assertion. In order to make a rejection, the examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. *In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993) (examiner must provide a reasonable explanation as to why the scope of protection provided by a claim is not adequately enabled by the disclosure). A specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as being in compliance with the enablement requirement of 35 U.S.C. 112, first paragraph, unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. Assuming that sufficient reason for such doubt exists, a rejection for failure to teach how to make and/or use will be proper on that basis. *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971). As stated by the court, "it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own ***with acceptable evidence or reasoning which is inconsistent with the contested statement.*** Otherwise, there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure." 439 F.2d at 224, 169 USPQ at 370 (emphasis added). The law regarding enablement fully supports Appellants' position. The law is clear that applicants do not have to submit proof of operability

to comply with the enablement requirement, and is equally clear that the absence of such proof alone is insufficient to meet the Office's burden.

Claims 32-34 refer to methods of inhibiting lymphocyte activation. The steps set forth in independent claim 32 include the step of "obtaining isolated Vpr protein" and the step of

> contacting lymphocyte cells with an amount of said Vpr protein effective to inhibit activation;
>
> wherein cytokine production and secretion by immunoglobulin activation of lymphocyte cells is inhibited.

In describing the methods, the specification clearly discloses that Vpr prevents lymphocyte activation. For example, on page 9 lines 11-14 of the specification states:

> It has been discovered that HIV protein vpr inhibits cell proliferation, induces undifferentiated cells to differentiate, that vpr effects modifies the state of macrophage cells, and prevents activation of lymphocytes.

On page 10, lines 14-20, the specification clearly describes what happens when activation is inhibited or prevented, stating

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In the Official Action dated February 20, 2008, the claims were rejected under 35 U.S.C. 112, first paragraph, for failing the enablement requirement. The Office incorrectly characterized the content of the specification as providing "no guidance regarding practice of the claimed methods" (page 5). On page 5, the Office erroneously concludes that the mechanism of action of Vpr in cells which is disclosed in the specification - that is the interaction of Vpr and

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Nothing in these three references establish any unpredictability with respect to the claimed invention. The specification is in agreement with the teachings of Mosmann, 1997 which describe the association of secretion of cytokines following activation of T cells. Likewise, nothing in Paul, 1987, contradicts the assertion that Vpr can prevent B lymphocyte activation. Finally, Rogel, February 1995, discloses that Vpr has biological activity although their observation were limited to linking Vpr expression with preventing cell proliferation; they did not study the effect of Vpr on lymphocyte activation. Accordingly, the reference did not make a reasoned argument why Appellants assertions of enablement should be questioned and the evidence they cited in support of the rejection did not support any assertion of unpredictability.

In Appellants' response filed August 19, 2008, Appellants pointed out that the Office had failed to establish a *prima facie* case of enablement. Having no obligation to do so, Appellants pointed out the relevant passages of the specification that describe lymphocyte activation and glucocorticoid receptor complex function disclosed above. Such response should have been sufficient to have the rejection withdrawn or provided anew with reasoning and support sufficient to meet the Office's burden.

Instead, in the Official Action dated November 19, 2008, the claims were rejected under 35 U.S.C. 112, first paragraph, for failing the enablement requirement. The Office restated the erroneous conclusion provided in the earlier Official Action, stating on page 4 that:

> [t]he disclosure fails to provide any guidance pertaining to the structural characteristics or mechanism of the interaction between Vpr and lymphocytes. The specification specifically discloses in more details and in working examples the use of Vpr or Rip-1-binding fragments of Vpr protein as transfection agent [sic] for the delivery of conjugated nucleic acid molecule [sic] or derivatives into the nucleus of a cell (page 36-37), which is not remotely related to inhibition or prevention of lymphocyte activation in vitro or in vivo, especially inside humans.

The rejection as set forth in the November 19, 2008 is improper in that it does not provide the requisite reasoning and evidence to support an initial conclusion of non-enablement. Rather, the Office focuses on a description of certain activities of Vpr directed at other claims not pending. The passages noted in the Official Action in no way contradict the claimed invention but rather are simply directed to other subject matter. The inclusion in the specification of a detailed description of a mechanism of action of another feature of Vpr does not undermine the assertion of enablement. More importantly, there is no requirement to provide a description of the mechanism by which the claimed invention works.

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> The Office has failed to set forth any reasoning or evidence to support the rejection. The Office has not established that the claimed invention does not meet the enablement requirement. Failing to do so, the burden is not properly shifted to Applicants. Applicants respectfully urge that the evidence and reasoning in the specification supports the conclusion that one skilled in the art would accept Applicant's assertion that the claims are enabled by the specification. In the absence of any evidence

> and reasoning in support of the rejection, Applicants are not required to put forth any evidence.
>
> Applicants respectfully request that any rejection based upon the Office statement above be withdrawn as the statements are not an accurate representation of the content of the specification. The Office has failed to provide any objective evidence or reasoning to question the validity of the contents of the specification. The Office has failed to present a single cited reference that demonstrates the claimed invention would not work. The Office has, instead, chosen to assert conclusory remarks about what one of ordinary skill in the art would not know based upon a negative inference drawn from references that do not discuss Vpr activity. The Office reasoning is therefore flawed and the evidence provided by the Office is not relevant.

The claims are fully enabled. There is no reasoning provided by the Office or evidence of record that suggests the Appellants assertions are in objective doubt. A simple, unsupported conclusion does not negate the Appellants position. A simple conclusion unsupported by any objective reasoning n of evidence is insufficient to establish a *prima facie* case of non-enablement. The Office has failed to fulfill their burden of proving that the specification does not enable the claimed invention.

Appellants vigorously urge that the rejection should be reversed because the Office has failed to meet its burden. The references cited by the Office do not establish the unpredictability in the field. On the contrary, they are wholly consistent with the enablement of the invention. The absence of working examples and the absence of a disclosure of the molecular mechanisms involved together with the cited references simply does not establish that the claimed invention is not enabled. Accordingly, the burden has not shifted to Appellants and the assertions of enablement should remain accepted.

Although unnecessary to teach how to make and use the claimed invention, the specification does indeed describe a mechanism of action involving Vpr which provides a

consistent explanation for the prevention of lymphocyte activation by Vpr. The glucocorticoid receptor complex is commonly known to inhibit the function of cytokine transcription. The specification clearly and expressly teaches that HIV Vpr interacts with Rip-1, a protein part of a the glucocorticoid receptor complex. The specification specifically discloses that Rip-1 is found in lymphocytes (Specification, page 46, lines 25-37) and that Vpr interacts with Rip-1 (Specification, page 49, lines 1-21). The specification specifically discloses that steroids induce the translocation of Rip-1 across the nuclear membrane. (Specification, page 48, lines 31-37) while glucocorticoid receptor antagonists prevent such translocation. The specification notes that steroids are known to have immunosuppressive effects. The specification specifically discloses that Vpr binds to Rip-1 which is part of the glucocorticoid receptor complex and prevents translocation of the complex. Accordingly, the specification discloses that Vpr and steroids have a similar function of translocating the glucocorticoid receptor to the nucleus. The specification discloses that one of Vpr's functions is its function as a translocation factor for glucocorticoid receptor; steroids are also translocation factor for glucocorticoid receptor. Steroids are immunosuppressants and the translocation of the glucocorticoid receptor to the nucleus is linked to this activity.

The claimed invention involves the step of contacting lymphocytes with an amount of Vpr protein effective to prevent lymphocyte activation. The specification plainly and expressly discloses the well-known biological function of glucocorticoids and their effects on the immune system. The specification demonstrates that Vpr mimics the biological function of dexamethasone and hydrocortisone, well-known glucocorticoids. Thus, one skilled in the art would expect that Vpr, which has a function that is like the nuclear translocation function of steroids, would be immunosuppressive. Moreover, when all of the evidence of record is considered, one skilled in the art would conclude that there is no reason to doubt Appellants' assertions of enablement.

While acknowledging that there is no requirement under the law for a patent application to contain a working example, the Office has asserted that a working example is necessary in the instant case because the Office the Rogel et al. reference discloses that Vpr functions to inhibit

cell proliferation. The fact that a reference describes a function of Vpr other than that in the present invention is insufficient to establish that the state of the art and invention are unpredictable.

This reasoning is flawed. One skilled in the art would not consider the fact that Vpr can inhibit cell proliferation as somehow making an assertion that Vpr prevents lymphocyte activation unpredictable. The two functions are not contradictory. The Rogel reference is does not establish, as the Office incorrectly states, that the "prior art is unpredictable". Likewise the Paul et al. reference is not contradictory with a finding of enablement. Moreover, the Mosmann reference actually supports a finding of enablement. There is no evidence provided by the Office that contradicts the plain and express statements of the specification that Vpr prevents lymphocyte activation. There is no evidence of record that contradicts the Appellants' disclosure of contacting lymphocyte cells with an amount of Vpr effective to prevent lymphocyte activation or raises any doubts as to why Appellants' assertions should be questioned.

Having submitted no reasoning or evidence that would cast objective doubt on the truth of the Appellants' statements in the specification, the Office has failed to sufficiently satisfy its burden to demonstrate that the claims are not enabled. When viewing the evidence of record in total, the evidence highly weighs in favor of finding the claims sufficiently enabled by the specification.

One skilled in the art would not conclude that the Office has met its evidentiary burden with respect to the enablement rejection. The rejection of claims 32-34 as failing the enablement requirement is erroneous. For the forgoing reasons, Appellants respectfully request that the rejection of claims 32-34 as being unpatentable under 35 U.S.C. § 112, first paragraph, as allegedly failing the enablement requirement, be reversed.

For the foregoing reasons, Appellants respectfully urge that the rejection of claims 21 – 23 and 32 – 34 under 35 U.S.C. § 112, first paragraph be reversed.

Respectfully submitted,

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Date: October 19, 2009

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(viii) Claims Appendix

The following claims are at issue in this appeal.

1. – 20. **(Canceled)**

21. A method of preventing lymphocyte activation which comprises the steps of:

obtaining isolated HIV Viral Protein R (Vpr) protein; and

contacting lymphocyte cells with an amount of said Vpr protein effective to prevent activation.

22. The method of claim 21 wherein said lymphocyte cells are T cells.

23. The method of claim 21 wherein said lymphocyte cells are B cells.

24.– 31. **(Canceled)**

32. A method of inhibiting lymphocyte activation which comprises the steps of:

obtaining isolated Vpr protein; and

contacting lymphocyte cells with an amount of said Vpr protein effective to inhibit activation;

wherein cytokine production and secretion by immunoglobulin activation of lymphocyte cells is inhibited.

33. The method of claim 32 wherein said lymphocyte cells are T cells.

34. The method of claim 32 wherein said lymphocyte cells are B cells.

(ix) Evidence Appendix

In support of section (vii) regarding the Argument, Applicants submit and attach hereto a copy of the following references cited by the examiner during prosecution and identified by where in the record the evidence was entered by the examiner:

William E. Paul, *et. al.*, Regulation of B-Lymphocyte Activation, Proliferation, and Differentiation, *Annals New York Academy of Sciences*, 1987;505:82-9, was cited by the examiner on page 5 of the Non-Final Office Action issued on February 20, 2008. Paul was cited again by the examiner on page 4 of the Final Office Action issued on November 19, 2008.

Tim R. Mosmann, *et. al.*, Functions of CD8 T-cell subsets secreting different cytokine patterns, *Immunology*, 1997; 9:87-92, was cited by the examiner on page 5 of the Non-Final Office Action issued on February 20, 2008. Mossmann was cited again by the examiner on page 4 of the Final Office Action issued on November 19, 2008.

Mark E. Rogel, et. al., The Human Immunodeficiency Virus Type I vpr Gene Prevents Cell Proliferation During Chronic Infection, Journal of Virology, Feb. 1995; 69(2): 882-888, was cited by the examiner on page 6 of the Non-Final Office Action issued on February 20, 2008. Rogel was cited again by the examiner on page 4 of the Final Office Action issued on November 19, 2008.

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The Human Immunodeficiency Virus Type 1 *vpr* Gene Prevents Cell Proliferation during Chronic Infection†

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Human immunodeficiency virus type 1 (HIV-1) is a retrovirus that can cause extensive cytopathicity in T cells. However, long-term productive infection of T-cell lines has been described. Here we show that although Vpr has no effect on the initial cytopathic effect of HIV-1, viruses that contain an intact *vpr* gene are unable to establish a chronic infection of T cells. However, virus with a mutated *vpr* gene can readily establish such long-term cultures. The effect of Vpr is independent of the *env* gene and the *nef* gene. Furthermore, expression of Vpr alone affects the progression of cells in the cell cycle. These results suggest that HIV-1 has evolved a viral gene to prevent chronic infection of T cells.

AIDS is characterized by a progressive depletion of CD4-positive lymphocytes. Immune system-mediated mechanisms of cell killing were proposed to explain the depletion of CD4 cells because the number of cells in the peripheral blood that were productively infected with human immunodeficiency virus (HIV) was not initially thought to be large enough to account for the cell loss. However, the large viral burden in lymph nodes (11, 28) and the fact HIV is very cytopathic in this setting (6) suggest that direct cell killing by the virus itself could play a considerable role in CD4 depletion.

Infection of susceptible cells by the cytopathic oncoretroviruses has been divided into an acute stage and a chronic stage (reviewed in reference 35). In the acute stage, these viruses cause extensive cytopathic effect that is accompanied by high levels of proviral DNA and superinfection of cells. After this, a chronic infection characterized by immunity to superinfection, no signs of cytopathic effect, and small numbers of proviruses per cell is established (36). HIV also causes cytopathic effect during acute infection and under some conditions can cause chronic infections. Cell death in the acute stage of infection is due to the envelope gene products (16, 21, 33, 39) and appears to be mediated by apoptosis (17, 21, 22, 37).

A previous report suggested that the ability of HIV-infected cell cultures to survive the acute infection and establish cell lines that produce viruses chronically is not mediated by the *env* gene but, rather, by the activity of accessory genes including *vpr* (25). Vpr is a 97-amino-acid protein that associates with the virion via interactions with the 3′ end of Gag (9, 18, 29, 40) and also accumulates in the nucleus of infected cells (20). Mutations in the HIV-1 *vpr* gene significantly reduce infection of terminally differentiated macrophages but have little or no effect on infection of rapidly dividing cells (4, 13, 38). Vpr and the matrix protein (MA) appear to be part of the preintegration complex that allows HIV proviral DNA to enter the nucleus in the absence of mitosis in some nonproliferating cells, such as macrophages (13).

Here, we show that Vpr of HIV-1 essentially prevents the chronic stage of infection of cells by HIV-1 but has no effect on the cytopathicity of the acute infection. Cell cultures infected with an HIV-1 viral stock that contains a full-length *vpr* gene eventually die out after infection. However, the same cultures infected with virus that is mutated in *vpr* recover from the initial cell death in the culture and eventually begin to replicate with the same doubling times as uninfected cells. Cells that eventually grew out of cultures infected with wild-type virus contained a mutated *vpr* open reading frame. In addition, we show that the effect of Vpr is independent of multiplicity of infection and, in contrast to a previous report (25), is independent of the presence of Nef. Furthermore, we show that the effect of Vpr late in the virus life cycle is independent of expression of the envelope gene products in peripheral blood mononuclear cells (PBMC). Transfections with plasmids that express Vpr alone indicate that Vpr expression can alter the progression of cells in the cell cycle and therefore may mediate its effect through prevention of cell proliferation.

MATERIALS AND METHODS

Cells. MT4 and SupT1 cells were obtained from the American Type Culture Collection and were grown in RPMI with 10% cosmic calf serum (Gibco). Peripheral blood lymphocytes were purified from buffy coat preparations, stimulated for 4 days with 0.9 μg of PHA-P (Difco) per ml, and grown in RPMI with 10% fetal bovine serum and 10% interleukin-2 (Cellpro).

Plasmids. All viruses are based on pLai (31), which contains open reading frames for all of the accessory proteins. Plasmid pLai was modified by addition of the simian virus 40 origin of replication in the plasmid backbone to increase virus production after transduction of cells containing the simian virus 40 T antigen (not shown). The following proviral mutants were used in this study: Vpr (insertion of 4 nucleotides at the *Nco*I site at position 5207), Nef (insertion of 4 nucleotides at the *Xho*I site at position 8490), MA NLS mutant (change of amino acids 26 and 27 of MA from Lys-Lys to Thr-Thr) (4), and Env (deletion between *Bgl*II sites at positions 6634 and 7214). Combinations of Vpr, Nef, Env, and MA NLS mutants were made by exchanging DNA fragments between the single mutants.

Virus and infections. Virus stocks were prepared by transfection of 293T cells (30) with 2.5 μg of proviral DNA per well of a six-well dish by the modified calcium phosphate method (8). Cell-free supernatant was collected 1 or 2 days after transfection, and the infectious titer was determined by the MAGI assay as described previously (15). Titers were typically 5×10^5 to 2×10^6 infectious units/ml.

Equal numbers of infectious units of virus were used to infect lymphoid cells. Typically, 1.5×10^6 cells were resuspended in 500 μl of media containing 1.5×10^5 infectious units of virus and 5 μg of Polybrene (Sigma). After 2 h at 37°C, the cells were pelleted, washed three times to remove residual virus, and resuspended in 2 ml of RPMI containing 10% fetal bovine serum. Viable cells were counted every 2 to 4 days based on trypan blue exclusion. $p24^{gag}$ was measured by enzyme-linked immunosorbent assay (ELISA; Coulter) with standards provided by the manufacturer.

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† Dedicated to the memory of Howard M. Temin.

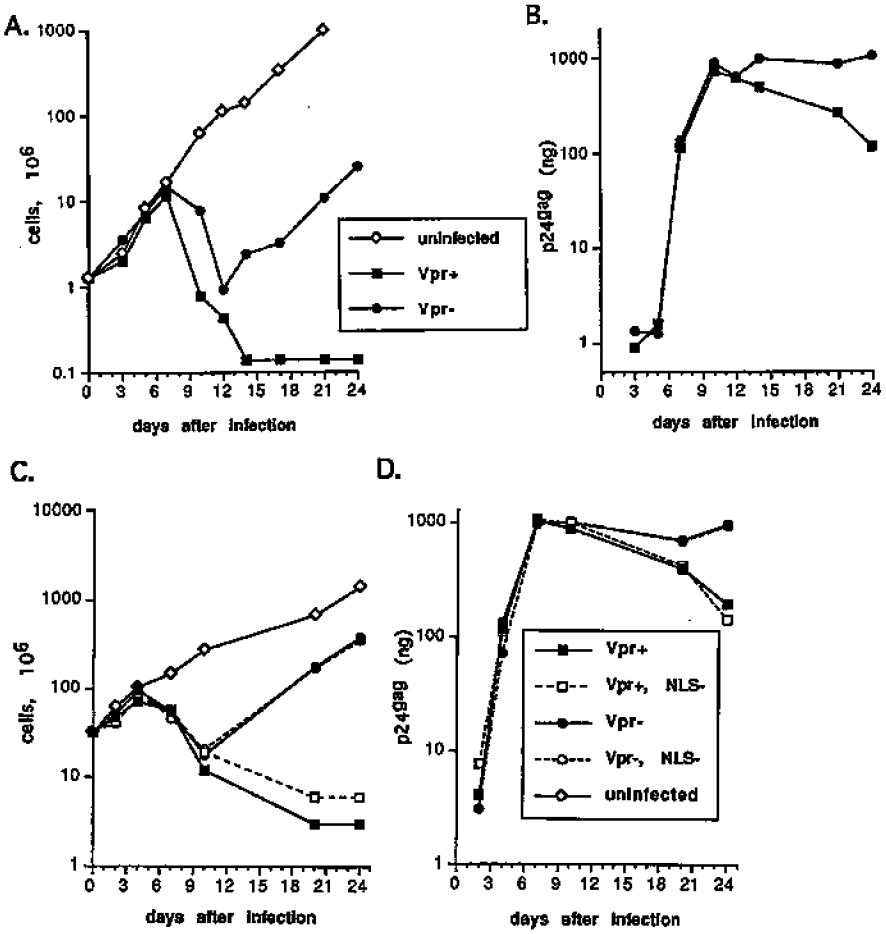

FIG. 1. Chronic infection is prevented in the presence of an intact *vpr* gene. SupT1 (A and B) or MT4 (C and D) cells were infected with HIV-1_{lai} with (Vpr^+) or without (Vpr^-) a full-length *vpr* gene at a multiplicity of about 0.1. The number of viable cells was determined by counting cells that excluded trypan blue (A and C), and the amount of $p24^{gag}$ was determined by ELISA (B and D). The number of cells is cumulative and was corrected for cells discarded when the culture was split (typically 1 to 2 every 3 days). Symbols as indicated in the figure are for panels A and B. The symbols for panels C and D are the same as in panels A and B, except that open squares are Vpr^+ NLS^- and open circles are Vpr^- NLS^-. NLS^- is the mutation in the nuclear localization sequence in MA as described previously (13).

Transient expression of Vpr and flow cytometry. Flow-cytometric analysis of whole cells was done as described previously (2) with some modifications. 293T cells were seeded to 1.5×10^5 per well in six-well plates. Transfections were carried out 2 days later with 5 μg of plasmid LTR-GFP (7), 0.6 μg of cytomegalovirus Tat, and 1 μg of either long terminal repeat (LTR) Vpr or LTR–NLS–β-galactosidase (15). At 48 h later, the cells were washed once with phosphate-buffered saline (PBS), removed from the plate with trypsin, diluted into 5 ml of PBS, and pelleted. They were then fixed in 1 ml of 2% formaldehyde at room temperature for 10 min, pelleted, and resuspended in 80% ethanol for 30 min on ice. After being washed, the cells were treated with 180 U of RNase A per ml in 1 ml of PBS at 37°C for 25 min, washed again, and then passed through a Nytex filer. Propidium iodide was added to 50 μg/ml, and the cells were incubated on ice for 1 h in the dark before they were analyzed by cell sorting.

Green fluorescent protein (GFP) expression was determined by fluorescence emission at 530 nm after excitation at 488 nm (7). Cells were gated positive for GFP expression on an FL2-versus-FL1 graph if the FL1 value was greater than the background determined by using mock-transfected cells after elimination of doublets. The DNA profiles and inferred G_1, S, and G_2 peaks were analyzed by the Multicycle AV program (Phoenix Flow Systems, San Diego, Calif.).

RESULTS

The presence of the Vpr prevents recovery of susceptible T-cell lines from the initial cytopathic effect of HIV-1 infection. To determine the long-term effect of Vpr expression on cells, we infected SupT1 cells (14), a T-cell line that is highly susceptible to the cytotoxic effect of HIV-1, with isogenic strains of HIV_{lai} that contain either a full-length *vpr* gene (called Vpr^+ here) or a truncated *vpr* gene that could encode a 45-amino-acid protein (called Vpr^- here). The number of viable cells in the culture were counted every 2 to 4 days for about 3 weeks, and cell-free supernatant was collected to determine the amount of virus produced.

In the initial week after infection, there was little difference between cells infected with Vpr^+ or Vpr^- virus in either virus growth or cell number (Fig. 1A and B). Large syncytia were observed in both cultures. Between days 7 and 12 after infection, the cell number declined dramatically in both infected-cell populations compared with the uninfected cells (Fig. 1A). However, after 12 days of infection there was a remarkable difference in the number of cells in the Vpr^+ infected culture and the number in the Vpr^- culture. By this time, cultures infected with Vpr^- virus had recovered from the initial cell death and began to grow with about the same doubling time as the uninfected cultures, while the cultures infected with wild-type virus did not recover (Fig. 1A). The amount of virus in the Vpr^- culture also remained at a plateau level, while the amount of virus in the Vpr^+ culture declined (Fig. 1B). This

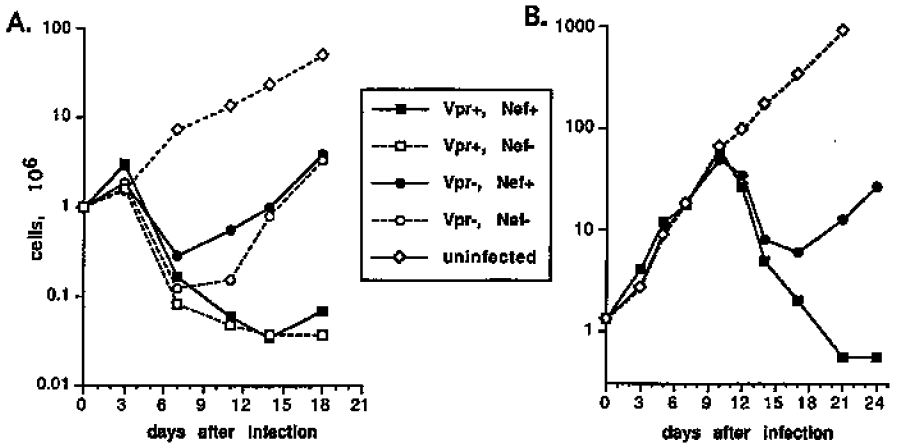

FIG. 2. The phenotype of Vpr late in infection is independent of the multiplicity of infection and of Nef. SupT1 cells were infected with $HIV\text{-}1_{lai}$ at a multiplicity of infection of either 1.0 (A), or 0.01 (B). Cells were counted by trypan blue exclusion every 2 to 4 days. Symbols are as indicated in the figure.

result suggests that the presence of Vpr prevents establishment of chronic infection in SupT1 cells.

MT4 cells are another T-cell line that is very sensitive to the cytopathic effects of HIV-1. MT4 cells differ from SupT1 cells in that unlike SupT1 cells, they do not form large syncytia after infection by the Lai strain of HIV-1. Therefore, we repeated the infections with MT4 cells and the same stocks of Vpr^+ and Vpr^- virus and counted cells periodically. The results with MT4 cells were very similar to those with SupT1 cells (Fig. 1C). That is, there was little difference in virus production between Vpr^+ and Vpr^- virus early after infection (Fig. 1D), and cells in cultures infected with either Vpr^+ or Vpr^- viruses began to die at similar rates up to 10 days after infection (Fig. 1C). However, by day 14, there were few cells remaining in the cultures infected with wild-type virus whereas the cultures infected with Vpr^- virus began to divide with the same doubling time as the uninfected cells (Fig. 1C).

Mutations in the MA NLS (4) have a similar phenotype to *vpr* mutants in that both the MA NLS and Vpr play a role in the efficient replication of HIV in macrophages (13). Therefore, to determine if mutations in MA acted similarly to mutations in *vpr* with respect to cell death, we also infected MT4 cells with Vpr^+ and Vpr^- viruses that contained a mutation in the MA NLS (Fig. 1C). Mutations in the MA NLS alone had no effect on the ability of Vpr to suppress cell growth late in infection and did not accentuate the Vpr^- phenotype (Fig. 1C). This indicates that cell death late in infection is not a property of genes that target the preintegration complex to the nucleus (13) but, rather, is specific to Vpr.

The experiments in Fig. 1 were done at a multiplicity of infection of about 0.1. By increasing or decreasing the multiplicity of infection, we could change the shape of the cell growth curve but could not change the final outcome (Fig. 2). That is, when the multiplicity was raised to about 1, cells began to die almost immediately after infection, although, again, eventually the cultures infected with Vpr^- virus recovered (Fig. 2A). The presence of an intact *nef* gene had no effect on this phenotype at either the higher multiplicity (Fig. 2A) or a lower multiplicity (data not shown).

When the multiplicity of infection was lowered to about 0.01, the infected cultures continued to divide at the same rate as uninfected cells for a longer period. However, eventually both Vpr^+ and Vpr^- cultures began to die, and this was followed by a period when only the cultures infected with Vpr^- virus grew out (Fig. 2B). These results indicate that the presence of the *vpr* gene in a provirus prevents cell growth during the chronic stages of virus infection in several cell lines and at different multiplicities of infection. In the absence of the *vpr* gene, cells that are normally highly susceptible to the cytotoxic effects of HIV-1 recover from the acute infection and establish chronically infected cultures that divide at nearly the same rate as uninfected cells.

Growth of cells infected with Vpr^+ virus is accompanied by mutation of the *vpr* open reading frame. In some cultures infected with Vpr^+ virus, cells eventually began to grow out and continued to produce virus. However, the recovery of the culture was always more slow than the recovery of cultures infected with Vpr^- virus (data not shown). We hypothesized that cells infected with Vpr^+ virus had incurred a mutation of the *vpr* reading frame that allowed the infected cells to recover. Therefore, MT4 cells that were infected with Vpr^+ virus (Fig. 1C) were kept in culture for an additional 2 months. Although there were few cells in the culture at the end of 3 weeks (Fig. 1C), by the end of 2 months the culture had recovered. Supernatant from the cultures were collected, and virus was partially purified by ultracentrifugation through a 20% sucrose cushion. Reverse transcriptase PCR with primers that flanked the *vpr* gene was then performed, and the PCR product was cloned and sequenced.

Two types of *vpr* clones were recovered from the chronically infected MT4 cells. In one class (half of the clones), the ATG start codon had been mutated to ACG. Because there is no other in-frame ATG codon in *vpr*, this mutation would effectively eliminate Vpr expression. The *vif* gene overlaps with *vpr* at the 5′ end of *vpr*. Therefore, it is noteworthy that the mutation of the start codon at the second base of the codon occurs in the third base of a *vif* codon and therefore would not change the *vif* reading frame. The second class of mutants contained point mutations in conserved regions of *vpr* (26), and the effects of these mutations on Vpr function is under investigation. In addition, it should be noted that the *vpr* alelles of two isolates of HIV-1 that had been recovered from chronically infected cells, the MFD isolate (34) and the HXB2 isolate (32), both contain frameshift mutations that cause premature truncations of Vpr after 72 and 42 amino acids, respectively (12). These data suggest that mutation of Vpr is required for con-

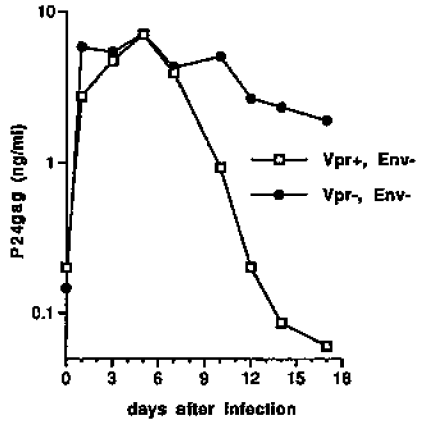

FIG. 3. Infection of PBMC with an HIV pseudotype. PBMC were infected with stocks of HIV that were created by transient cotransfection of an amphotropic envelope-expressing plasmid and an envelope mutant HIV provirus. The medium was completely changed every 2 to 4 days, and the level of $p24^{gag}$ was measured.

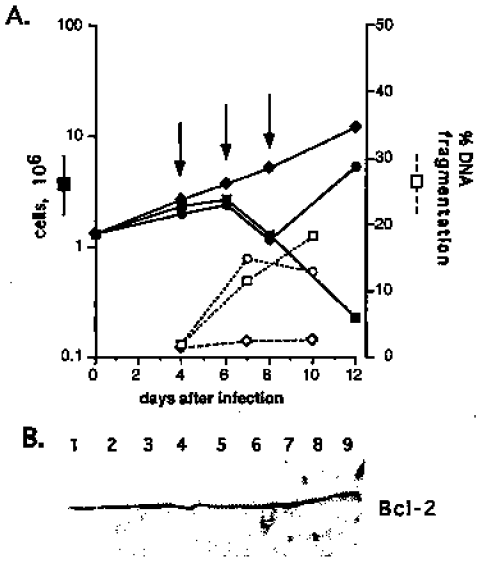

FIG. 4. Bcl-2 levels and apoptosis do not account for the Vpr phenotype. MT4 cells were infected with Vpr^+ or Vpr^- virus at a multiplicity of infection of about 0.1. (A) Viable cells were counted on days 4, 6, 8, and 12 by trypan blue exclusion (solid symbols, left *y* axis). About 10^6 cells were taken at days 4, 7, and 10 to determine the amount of intracellular fragmented DNA (open symbols, right *y* axis). Symbols: diamonds, uninfected cells; squares, cells infected with Vpr^+ virus; circles, cells infected with Vpr^- virus. (B) On days 4, 6, and 8 after infection (indicated by the arrows in panel A), equal cell equivalents of protein were loaded on a 12.5% polyacrylamide gel, blotted, and probed with antibody to Bcl-2. Lanes: 1, 2, and 3, extracts from cells infected with Vpr^+ virus at days 4, 6, and 8 days after infection, respectively; 4, 5, and 6; extracts from cells infected with Vpr^- virus at days 4, 6, and 8 days after infection, respectively; 7, 8, and 9, extracts from uninfected cells taken in parallel with those of the infected cells.

tinued growth of infected cells, although we cannot yet rule out additional changes in the provirus.

Vpr-induced cell death in the absence of Env in the context of a provirus. We wished to determine if Vpr expressed in the context of a provirus had an effect in PBMC cells in addition to its effect on T-cell lines. However, because it is difficult to culture primary T cells for long periods, we wished to carry out these experiments in the absence of spreading virus so that the effect of Vpr could be assessed without the necessity to establish a chronic infection. Therefore, HIV-1 pseudotypes that contained either a wild-type or a truncated *vpr* gene were prepared by cotransfection of HIV-1 molecular clones and an expression vector for the murine amphotropic envelope gene (27). In each case, the HIV proviruses contained a large deletion in the envelope gene such that gp120/gp41 could not be expressed (see Materials and Methods).

The titers of both Vpr^+ Env^- and Vpr^- Env^- viruses pseudotyped with amphotropic envelope was 2×10^5 infectious units per ml on HeLa-CD4/LTR–β-gal cells (15). PBMC were stimulated with phytohemagglutinin for 4 days to produce primarily activated T cells. Then, 2×10^6 cells were infected with 1 ml of virus, washed three times, and resuspended in new medium. Because the viruses used for these infections have a deletion in the envelope gene and are therefore capable of only one round of infection, the maximum number of cells infected is a small percentage of the population. Although we would be unable to detect a 10% decrease in cell number, we predicted that if proviruses with Vpr were cytotoxic in the absence of the envelope, the expression of the viral *gag* gene would decrease over time because of loss of infected cells from the culture.

Cell-free supernatant was collected from the infected PBMC 1 day after infection and every 2 or 3 days thereafter (Fig. 3). The day 0 collection was done immediately after the cells were washed after infection, and therefore it represents the residual input virus in the medium (Fig. 3). Each time the supernatant was collected, the cells were centrifuged, washed once, and resuspended in new medium. As expected, $p24^{gag}$ was found in the supernatant as early as 1 day after infection. The amount of $p24^{gag}$ in both Vpr^+- and Vpr^--infected cultures was stable for at least 1 week. However, by day 10, the expression of $p24^{gag}$ decayed significantly more rapidly in cultures infected with the Vpr^+ pseudotype than in cultures infected with the Vpr^- pseudotype (Fig. 3). These results indicate that viral expression is sustained for longer periods in the absence of Vpr than in the presence of Vpr in PBMC and are consistent with the hypothesis that the presence of Vpr inhibits the proliferation of infected primary cells.

The effect of Vpr is not mediated through Bcl-2 levels. Other investigators have noted that acute HIV-induced cytopathic effect is accompanied by markers of apoptosis (6, 17, 22, 37), and it has been suggested that HIV-1 infection can regulate Bcl-2 levels in B cells (10). Therefore, one hypothesis to explain the recovery from initial cell death of cultures of cells infected with Vpr^- virus is that levels of Bcl-2 were modified by Vpr, thereby affecting cell death later in infection.

To test this hypothesis, we examined both DNA fragmentation, a marker of apoptosis (23), and the amount of Bcl-2 protein in the infected cultures after infection (Fig. 4). As in previous experiments, the number of cells in the culture that had been infected with Vpr^- virus first decreased and then began to increase by day 12 (Fig. 4A). The presence of fragmented DNA on days 6 and 8 suggested that death occurred by apoptosis and correlated with decreases in cell number (Fig. 4A, open symbols). However, the amount of fragmented DNA did not predict which culture would recover from acute cell death.

There is a high level of endogenous Bcl-2 in MT4 cells (Fig. 4B, lanes 7 to 9), as well as in other T-cell lines that we examined (data not shown). On days 4, 6, and 8 after infection (arrows in Fig. 4A), equal amounts of total protein from the cell cultures were probed with Bcl-2 specific antibody. Although there is extensive cell death in the infected cultures, the

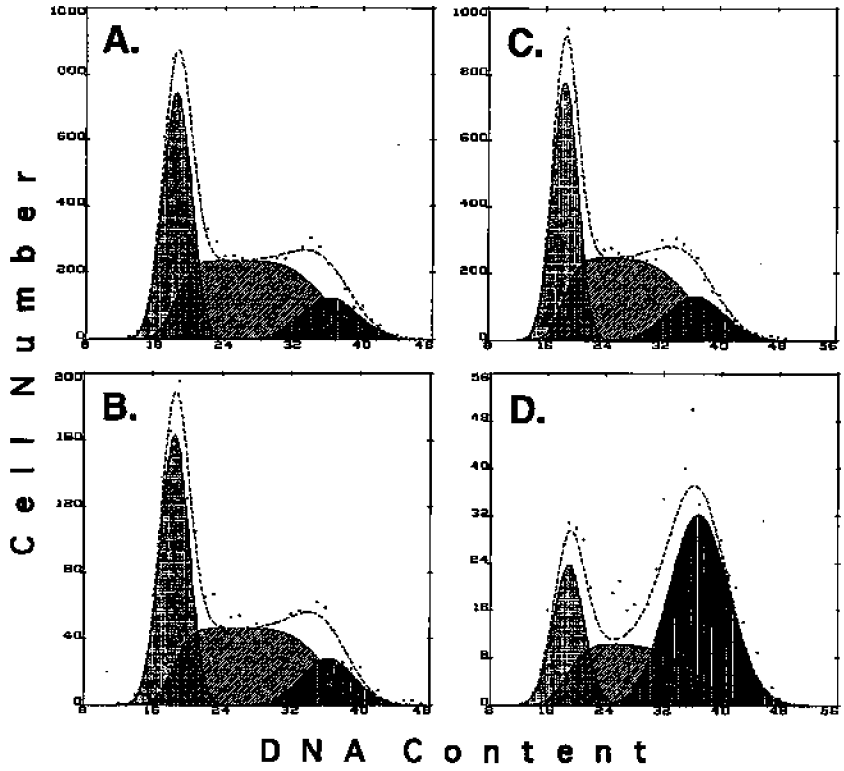

FIG. 5. Vpr transient transfection alters the cell cycle distribution. 293T cells were transfected with a plasmid that expresses the GFP and either Vpr (C and D) or a nucleus-localized β-galactosidase (A and B) from the HIV-1 LTR. The DNA profile of the cells that were GFP positive was analyzed separately from the DNA profile of the cells that were GFP negative. (A) GFP-negative population transfected with NLS–β-galactosidase. (B) GFP-positive population transfected with NLS–β-galactosidase. (C) GFP-negative population transfected with Vpr. (D) GFP-positive population transfected with Vpr. The G_1, S, and G_2 peaks are shown underneath the DNA profile.

amount of Bcl-2 does not significantly increase or decrease relative to that in the uninfected controls, except perhaps for a slight increase on day 8 (Fig. 4B, lanes 3 and 6). Furthermore, there was no difference in levels of Bcl-2 between the cultures that would recover from the initial cell death (the cultures infected with Vpr^- virus [lanes 4 to 6]) and the cultures which did not recover from the initial cell death (the cultures infected with the Vpr^+ virus [lanes 1 to 3]). Reprobing the blot with HIV serum revealed that the Vpr^- and Vpr^+ cultures contained equal amounts of viral protein on days 6 and 8 after infection (data not shown). These results indicate that the difference between culture death (Vpr^+) and culture survival (Vpr^-) is not modulated by differences in Bcl-2 expression. Furthermore, forced expression of Bcl-2 or Bcl-X_L (3) via retroviral vectors did not rescue cultures infected with wild-type Vpr^+ virus (data not shown). Therefore, these results do not support the hypothesis that the presence of Vpr in the provirus exerts its effect on viability of infected cells by modulating Bcl-2.

Expression of Vpr alone alters the distribution of cells in the cell cycle. We wished to determine if Vpr expression could affect cells directly by analyzing the effect of Vpr on cell cycle distribution of transfected cells. Therefore, we transiently cotransfected 293T cells with a plasmid that expresses Vpr from the HIV-1 LTR and with a plasmid that expresses GFP from the same promoter (the HIV-1 LTR) in order to mark successfully transfected cells. Transfected cells were fixed and stained with propidium idodide as whole cells to determine the DNA content and GFP expression simultaneously. As a control for the effects of high-level expression of an exogenous nuclear protein, cells were also cotransfected with GFP and an NLS β-galactosidase gene (15) under control of the HIV-1 LTR. All transfections included the Tat protein to achieve high-level expression from the HIV-1 LTR. Cells that expressed GFP (and therefore were presumed to express the cotransfected marker) were gated on the fluorescence-activated cell sorter, and the DNA profiles of both the GFP-positive populations (Fig. 5B and D) and the GFP-negative populations (Fig. 5A and C) were determined to compare transfected and nontransfected cells in the same culture.

The DNA profiles of GFP-negative populations transfected with either Vpr or β-galactosidase were nearly identical (Fig. 5, compare panels A and C). These cells are presumably nontransfected cells in the culture. The DNA profile of the cells that specifically took up and expressed DNA was then examined by gating the analysis on cells that expressed GFP (Fig. 5B and D). In the control, the GFP-positive cells in the culture cotransfected with both GFP and NLS–β-galactosidase had a DNA profile similar to that of the GFP-negative population (Fig. 5, compare panels A and B). In both populations, about 12% of the cells were in G_2. This indicates that expression of a transfected gene alone does alter cell cycle progression.

On the other hand, the GFP-positive cells that were also transfected with a Vpr expression vector had a DNA profile that was markedly skewed toward G_2 accumulation of DNA (Fig. 5, compare panels C and D). About 52% of the cells were

in G_2 in the GFP-positive population, compared with about 13% of the cells in the GFP-negative population in the same culture. These results demonstrate that expression of Vpr in those cells is capable of altering their cell cycle distribution.

DISCUSSION

We have shown that in the presence of an intact *vpr* gene, HIV-1 will kill an entire culture of infected cells. On the other hand, when *vpr* is mutated, the virus will initially kill a large proportion of cells, but a small proportion of the culture will eventually survive and become a chronic producer of virus. Selection for mutations of the *vpr* gene occurs in long-term cultures of cells initially infected with wild-type virus. These results indicate that Vpr has a role in preventing the establishment of cell cultures that chronically produce virus.

The effect of Vpr is observed late after infection even in peripheral blood lymphocytes that were infected with a Vpr^+ provirus that could not encode envelope and therefore could not spread (Fig. 3). This result suggests that the effect of Vpr results from accumulation of newly synthesized Vpr from the integrated provirus rather than from Vpr that is brought into the infected cells in the virion (9) and present in the preintegration complex (13). In addition, it is doubtful that Vpr is responsible for the acute cell death, since cultures infected with either Vpr^+ or Vpr^- virus initially die at equal rates (Fig. 1 and 2) with signs of apoptosis (Fig. 4). We presume that gp120/gp41 is responsible for the acute cytopathicity. A minor effect of Nef on cytopathic effect (25) also cannot be excluded from our data.

The mechanism of action of Vpr is unclear. Vpr itself could be cytotoxic late in infection. However, the effect of Vpr was independent of Bcl-2 and BclX. Furthermore, it had previously been shown that Vpr could cause terminal differentiation of rhabdosarcoma cells (19). Our results are therefore probably more consistent with a hypothesis that Vpr is cytostatic and therefore prevents cells from continued proliferation (or causes their terminal differentiation). On the other hand, Vpr could be specifically toxic to cycling cells. The effects of transfection of Vpr alone (Fig. 5) are consistent with the hypotheses that it produces an arrest of cell cycle progression so that cells accumulate in G_2. A cytotoxic effect of Vpr only in cycling cells would explain why primary macrophages, which are withdrawn from cycling because of differentiation, are capable of long-term expression of HIV-1 without major cytotoxic effects (24), whereas PBMCs are rapidly killed by the virus in a productive infection.

The major reservoir of infected cells in vivo contains latent rather than active proviruses (1, 5, 11). Therefore, the fact that Vpr prevents establishment of chronic infections in T cells may have only a minor effect on total virus burden, although it may contribute to the ultimate decline in CD4 cells. Therefore, the importance of Vpr in macrophages (13, 38) alone could ensure its continued selection in vivo. On the other hand, there may be an evolutionary advantage for HIV to prevent replication of infected T cells. For example, $CD4^+$ cell clones that are specific for HIV antigens would be prevented from expansion and participation in cytotoxic T-lymphocyte responses after infection. The effect of Vpr on the cell cycle also raises the possibility that it plays a role in the differentiation of cells in vivo.

ACKNOWLEDGMENTS

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Functions of CD8 T-cell subsets secreting different cytokine patterns

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CD8⁺ T cells can differentiate into two effector phenotypes, Tc1 and Tc2, secreting different cytokine patterns. Both subsets are cytotoxic via the perforin and Fas pathways, and both kill resting and activated B cells, ruling out the possibility of cognate help, although Tc2 cells may provide bystander help. Both subsets induce inflammation with similar cellular infiltrates. Tc1 cytokine synthesis is limited by two mechanisms — IL-4 induces a permanent deficiency in cytokine secretion, and rapid killing of target cells limits CD8⁺ T-cell activation and cytokine production. These multiple CD8 T-cell activities provide a versatile set of immune functions.

Key words: anergy / cytokines / Tc1 / Tc2 / T-cell differentation / Th1 / Th2

CD8⁺ T CELLS RECOGNIZE antigen peptides presented on MHC class I, and kill the antigen-bearing cells. As MHC class I molecules contain mainly peptides derived from the cytosol, this is an effective mechanism for killing cells infected with viruses or other intracellular pathogens. However, CD8⁺ T cells have also been associated with suppression of immune responses, and their ability to produce various cytokines suggests additional functions. The recent discovery of subsets of CD8⁺ T cells producing different patterns of cytokines suggests that different functions may be mediated by each subset.

Among CD4⁺ effector T cells, the Th1 and Th2 cytokine secretion patterns have been associated with very different functions.[1] Th1 cells and their cytokines are often associated with delayed type hypersensitivity (DTH) reactions, and the Th1 cytokine interferon γ (IFNγ) is a potent activator of macrophage effector functions. Th2 cells and their cytokines are often associated with strong antibody responses, and particularly with allergic reactions and anti-helminth responses. Although other cytokine patterns have also been identified. Th1 and Th2 are two major cytokine patterns that regulate strongly polarized sets of effector functions. During many infections, the choice of a Th1 or Th2 response is crucial for the outcome of the host defence against infection,[2] e.g. a Th1 response is often essential for clearance of intracellular pathogens such as Leishmania major, and a Th2 response is most effective against some helminths.[3]

The major cytokine pattern expressed by CD8⁺ effector T cells is similar to the Th1 set of cytokines.[4,5] The production of IFNγ, tumor necrosis factor and lymphotoxin by CD8⁺ cells is consistent with their direct cytotoxic function, as these three cytokines activate cytotoxic functions in macrophages and granulocytes. However, not all CD8⁺ cells secrete a Th1-like pattern. CD8⁺ T-cell clones can secrete both Th1 and Th2 cytokines.[6] Some CD8⁺ T-cell clones derived from lepromatous leprosy patients secreted interleukin 4 (IL-4) but only low amounts of IFNγ.[7] After treatment with PMA and ionomycin, CD8⁺ T cells lost expression of CD8⁺, became non-cytotoxic, and secreted IL-4.[8] Several groups then showed that CD8⁺ T cells could express a Th2-like cytokine pattern when differentiated *in vivo*[9] (P. Openshaw, personal communication) or *in vitro*.[10-12] We have been interested in the functions of the different subsets of CD8⁺ T cells, particularly because the effector functions of CD4⁺ Th2 cells and their cytokines (B-cell help, allergy) did not appear to fit well with the cytotoxic functions of CD8⁺ T cells.

CD8⁺ T-cell subsets — Tc1 and Tc2

For this article, Tc1 cells are defined as CD8⁺ T cells that secrete IFNγ but not IL 4 or IL 5, and Tc2 cells

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are $CD8^+$ cells that secrete IL-4 and IL-5 but not IFNγ. As described below, the synthesis of IL-2 by mouse Tc1 cells is subject to further regulation, and the Th2-specific cytokines IL-6 and IL-10 are synthesized in high amounts by Tc2 cells, but also in lower amounts by Tc1 cells. Thus the limited definitions above, based only on IFNγ, IL-4 and IL-5, are appropriate for $CD8^+$ T cells. It should be noted that the cytokine patterns of mouse $CD8^+$ T-cell subsets are similar to those of human CD4 and CD8 T cells, i.e. IL-4 and IL-5 are Th2/Th2 specific, whereas IL-6 and IL-10 are preferentially but not exclusively synthesized by Th2/Tc2 clones. Both Tc1 and Tc2 cells express GM-CSF and IL-3, although IL-3 levels are low, particularly in Tc1 cells. Activation of T cells by either Con A or antigen results in similar cytokine patterns.

Differentiation of $CD8^+$ T-cell subsets

Unlike mouse $CD4^+$ T cells, which can differentiate readily into either Th1 or Th2 cells, naive $CD8^+$ T cells show a strong preference for differentiating into Tc1 cells. As for differentiation of $CD4^+$ cells to the Th1 phenotype, IFNγ and IL-12 encourage differentiation to Tc1 cells. Tc2 differentiation requires substantial amounts of IL-4, and it is normally also necessary to prevent the action of IFNγ, e.g. with blocking antibody.[11] The antigen presenting cells (APC) can be important. The M12 B cell lymphoma induces good Tc2 differentiation in the presence of IL-4 and anti-IFNγ antibody, whereas the macrophage cell line J774 only supports Tc2 differentiation if IL-10 is also added to the cultures (T. Mosmann and W. Fu, unpublished).

Cytotoxicity

IL-4-secreting $CD8^+$ T cells can be less cytotoxic than Tc1 cells,[9] but they can also show similar cytotoxic ability,[12,13] probably depending on the conditions used for growth and maintenance. Both Tc1 and Tc2 cells kill mainly by the perforin pathway, as shown by the abrogation of killing if calcium is chelated or the T cells are derived from perforin-deficient mice. Both subsets also kill Fas-expressing target cells, particularly cells expressing transfected Fas at high levels.[13]

B-cell help

Although there have been reports that Tc2 cells can help B cells,[9,12] it is difficult to reconcile this function with the strong cytotoxic ability of Tc2 cells. We have examined Tc2 interactions with B cells in more detail, using T cells that specifically recognize alloantigens on target B cells. Both Tc1 and Tc2 cells efficiently kill either resting or activated normal B cells.[13] Th1 cells show weak killing, whereas Th2 cells show no killing, and even reduce background lysis of the B cells. In the absence of perforin, most of the killing of resting B cells by either Tc1 or Tc2 cells is abrogated. In contrast, activated B cells are still killed by either $CD8^+$ subset in the absence of perforin. This may occur via the Fas pathway, as B cells are susceptible to Fas-ligand-mediated death when activated in the absence of Ig receptor signalling.[14]

Using small resting B cells, allospecific Th2 cells provided strong help for IgM and IgG synthesis. Th1 cells provided moderate help, but Tc1 or Tc2 cells did not induce antibody production if they directly recognized alloantigen on the B cells. Similar experiments with T cells from perforin-deficient mice showed that Tc1 and particularly Tc2 cells could provide some help for IgM synthesis, but at lower levels than CD4 cells. Tc2 cells also induced synthesis of IgG1, in accordance with their production of the IgG1 switch factor IL-4. This help was only observed at higher T-cell numbers, and never reached the levels of IgG1 induced by Th2 cells.

In contrast to the outcome of direct T-cell recognition of B cells, some help could be provided if Tc2 cells were activated independently, by immobilized anti-CD3 antibodies.[11-13] As cytotoxicity is a directional, one-on-one process, the T cells would probably focus the killing mechanisms on the plate-bound side of the T cell. This is supported by the ability of immobilized anti-CD3 antibodies to reduce the killing of B cells by Tc2 cells.[13] The remainder of the T-cell surface may then be able to interact with the B cells, for example via CD40 ligand, which is expressed at low levels on Tc2 cells.[13] The large amounts of Th2-like cytokines produced by Tc2 cells would also augment this bystander helper effect.

Proliferation of B cells induced by allospecific T cells showed a similar hierarchy — Th2 > Th1 > Tc2 > Tc1.[13] Again, the absence of perforin improved the ability of the Tc cells to induce proliferation, but not to the levels induced by CD4 cells. Both the proliferation and antibody secretion data are consistent with the ability of even perforin-

deficient Tc1 or Tc2 cells to kill more efficiently than CD4 cells.

The inability of Tc1 or Tc2 cells to provide cognate help to B cells is consistent with the antigen processing pathways used for MHC class I and class II presentation. Antigen captured by B-cell surface antibody is processed and presented on MHC class II, whereas MHC class I presents peptides derived from proteins synthesised in the B cell. Although another pathway has recently been described,[15] in which exogenous antigen can be processed and presented on MHC class I, this pathway operates in phagocytes and a transfected B-cell tumor.[16] As the presentation of antibody-captured antigen on MHC class I would lead to killing of antigen-specific B cells, thus abrogating the specific antibody response, it appears unlikely that B cells would normally express this pathway. Thus by the regular antigen-processing pathways, foreign antigens would be presented on MHC class II if the B cell expressed antibody specific for that antigen, whereas the antigen would be expressed on MHC class I if the B cell was infected. Interaction with CD4 and CD8 cells, respectively, would ensure that antigen-specific B cells would be helped, whereas infected B cells would be killed. When Tc2 cells are activated by recognizing and killing infected cells (not necessarily B cells) the Tc2 cells will express cytokines and surface molecules that may provide antigen-non-specific help for neighbouring B cells, to augment the effects of antigen-specific Th2 cells.

CD8 cells and DTH

As Th1 but not Th2 cells can induce a DTH reaction when injected into mouse footpads,[17] and Th1 cytokines are often associated with DTH, we tested whether Tc1 and Tc2 cells showed a similar separation of function. Surprisingly, both Tc1 and Tc2 cells gave similar inflammatory reactions when injected into the footpads of mice expressing the alloantigen recognized by the T cells.[18] The Tc1 and Tc2 reactions were similar in the magnitude and kinetics of the swelling, and the extent of vascular leakage. Cellular infiltration was assessed by staining footpad sections with monoclonal antibodies, and by making single-cell suspensions and quantitating the number of infiltrating cells. Both Tc1 and Tc2 cells induced strong infiltration of cells expressing the Gr1 antigen, typical of neutrophils, and similar numbers of neutrophils were identified morphologically. Mac3+ cells were also recruited in similar numbers by either Tc1 or Tc2 reactions. Eosinophils were present in larger numbers in Tc2 reactions, particularly at later times, consistent with the eosinophil attractant, proliferation and survival effects of IL-4 and IL-5.

As the strong ability of Tc2 cells contrasted with the poor ability of Th2 cells to induce DTH in this model, we tested whether the cytokine expression patterns were altered *in vivo*. However, ELISA analysis of footpad extracts showed that IFNγ was expressed only in Tc1 reactions, whereas IL-4 and IL-5 were expressed only in Tc2 reactions.[18] Interestingly, both of the inflammatory cytokines IL-6 and TNF were expressed equally in Tc1 and Tc2 reactions, possibly because these cytokines were expressed mainly by secondary infiltrating cells such as macrophages.

Tc2 but not Th2 cells can kill target cells, raising the possibility that the death of substantial numbers of target cells in Tc2 reactions may have initiated the inflammatory reaction. However, Tc1 and Tc2 cells from perforin-deficient mice were still able to initiate substantial DTH reactions, although in some experiments the magnitude of the reaction was somewhat reduced.[18]

In this test system, effector T cells were injected directly into footpads, thus only the effector phase of the DTH reaction was tested. In order to test the relative abilities of Tc1 and Tc2 cells to home into sites of inflammation, radiolabelled Tc1 or Tc2 cells were injected i.v. into mice in which inflammatory reactions had been initiated in one footpad by the injection of unlabelled Tc1 or Tc2 cells. Both Tc1 and Tc2 cells showed selective localization into the footpad with inflammation, whether the reaction was induced by Tc1 or Tc2 cells.[18] Thus Tc2 cells can mediate both stages of the DTH reaction — selective migration to a site where inflammation is initiated, and recruitment of inflammatory effector cells, macrophages and granulocytes, to the site of inflammation.

Although DTH induction by Tc2 cells appears to run counter to the selective ability of CD4 Th1 cells to induce DTH, there is accumulating evidence that Th2-like cytokine patterns can also be associated with DTH reactions. Preactivated Th2 cells can induce inflammation that is dependent on IL-4.[19] In a granuloma model in which antigen-coated beads are localized in the lung, the mycobacterial antigen PPD induces granulomas that are associated with the production of Th1-like cytokines, and anti-IFNγ antibodies reduce the size of the granulomas.[20] More surprisingly, when similar experiments were carried out with a strong Th2-inducing antigen (schistosome

egg antigen, ref 21) conjugated to the beads, granulomas were also obtained, but the granuloma size was increased by treatment with anti-IFNγ, suggesting that the cytokine regulation was quite different between the two reactions.

Thus inflammatory reactions may be induced by more than one set of cytokine effects. It should be noted that the effector functions of infiltrating cells are affected strongly by Th1 cytokines (generally activating) and Th2 cytokines (mainly inhibitory). Thus the induction of inflammation by T cells secreting different cytokine patterns may allow different effector functions to be expressed at a site of infection, providing additional versatility in the immune response.

Further differenitation of Tc1 cells — 'anergy'?

Although Tc1 and Tc2 phenotypes do not seem to be interconvertible, Tc1 cells can undergo further differentiation. Treatment with IL-4, in either the presence or absence of APC, induces loss of the subsequent ability of the Tc1 cells to synthesize IL-2 in response to Con A or antigen.[22] This loss appears to be permanent, even in the absence of further IL-4 treatment. Synthesis of other cytokines is also impaired, particularly in response to antigen stimulation.[23]

This further differentiation of Tc1 cells may explain previous results suggesting that some but not all CD8 cells synthesize IL-2. Tc1 cells prepared in the presence of anti-IL-4 antibodies uniformly synthesize moderate amounts of IL-2. However, IL-2 production is still considerably lower than the levels produced by Th1 cells, so that CD8 cells may be a major source of IFNγ, but only a minor source of IL-2.

The cytokine-deficient Tc1 cells induced by IL-4 treatment show unimpaired cytotoxicity by either perforin or Fas pathways.[22] Surprisingly, the ability to induce DTH is also retained, although the cytokine-deficient cells produce IFN_γ at much lower levels *in vivo* during the DTH reaction.[23]

As a consequence of the loss of ability to produce IL-2, the cytokine-deficient Tc1 cells cannot sustain independent proliferation in response to APC, although they proliferate normally if exogenous IL-2 is added to the cultures. This behaviour is similar to the state of 'anergy' that has been described in CD4 and CD8 cells stimulated in the presence of reduced costimulation.[24,25] However, 'anergy' may not be an appropriate description of cells that retain strong cytotoxicity, induce DTH, and proliferate extensively in response to antigen plus IL-2.

This effect of IL-4 on Tc1 cells could be another example of cross-regulation of type 1 and type 2 cytokine patterns.[1] As a consequence of IL-4 activity, the Tc1 cells would retain their immediate effector functions. However, they would not be capable of sustaining independent proliferation, but would instead be dependent on IL-2 produced by other cells, e.g. Th1 cells. This would impair long-term responses, as shown by incubation of low numbers of normal or cytokine-deficient Tc1 cells with proliferating tumour target cells. As expected, even though both types of Tc1 kill equally in the short-term, only normal, IL-2-secreting Tc1 cells can proliferate and deal with large numbers of tumour target cells in longer-term assays.[23]

Cytotoxicity limits cytokine synthesis

CD4 cells are often considered to be the major cytokine producers, and when stimulated by low or moderate numbers of APC, Th1 cell populations *in vitro* produce much higher levels of IL-2 and IFNγ than Tc1 cells. However, CD8 clones and populations stimulated with Con A or high APC numbers *in vitro* produce some cytokines, e.g. IFNγ, at levels equal to those produced by CD4 T cells. This apparent paradox is resolved by considering the different effects of CD4 and CD8 cells on APC. $CD8^+$ T cells but not $CD4^+$ T cells kill APC, thus limiting the duration of stimulation, and also the availability of APC for stimulating other CD8 cells. This model was confirmed by using perforin-deficient Tc1 cells, which produced much higher levels of cytokines than normal Tc1 cells, at lower APC numbers.[26] The alternate Fas ligand killing pathway can also contribute to reduced cytokine levels, as perforin-deficient Tc1 cells produce lower cytokine levels in response to targets expressing high levels of Fas. As high numbers of APC induce high levels of cytokine production from even fully cytotoxic CD8 cells, frequent stimulation with multiple targets may substitute for the sustained stimulation by a single target received by CD4 T cells.

Two mechanisms may thus explain why CD8 cells are dependent on $CD4^+$ T-cell help in some systems but not others. Exposure to IL4, or activation by APC with insufficient costimulation, may induce loss of the ability of $CD8^+$ T cells to secrete IL-2. Alternatively, the synthesis of IL-2 may be low in circumstances

where the $CD8^+$ T cells are stimulated infrequently. In either case, the $CD8^+$ T cells may then be dependent on IL-2 derived from $CD4^+$ T cells.

The variable cytokine production of CD8 T cells may be important at different stages of the immune response. Initially, CD8 precursor cells are non-cytotoxic, so that they would not be expected to kill their APC, allowing optimal stimulation and proliferation. A few days later, the CD8 cells have differentiated into cytotoxic effectors, which can migrate to the site of infection and kill infected target cells. If infected cells are abundant, it would be expected that the CD8 cells would secrete large amounts of cytokines, augmenting the inflammatory reaction and recruiting additional specific and non-specific effectors to the site. As the infection is brought under control, the number of infected targets will decrease, so that CD8 cells may still kill the remaining infected cells, but this will occur sporadically, and the in-vitro results suggest that this would not result in substantial cytokine synthesis. This may provide a mechanism for tapering off the potentially damaging inflammatory response as an infection is resolved.

Conclusion

The various aspects of CD8 T-cell function described above can all contribute to the diversity of effector functions available during an immune response. For example, Tc2 cells may allow strong cytotoxicity to co-exist with strong Th2-mediated antibody responses, whereas Tc1 and Th2 responses would cross-inhibit due to conflicting cytokine patterns. Similarly, inflammatory reactions mediated by Tc2 and Tc1 cells may show differences in the effector functions of the infiltrating cells, so that the two types of response are appropriate for different pathogens. Cytotoxic and pro-inflammatory functions of CD8 cells may be separately regulated depending on the frequency of infected targets. Thus the immune system has a versatile array of effector functions, which should allow an optimal mixture of effector functions to be chosen to effectively attack each pathogen. Precise matching of effector functions with pathogens is important not only to attack the infecting organism with the most effective mechanisms, but also to avoid immunopathology caused by effector mechanisms that do not contribute to pathogen destruction.

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PART II. IMMUNE PATHOGENESIS OF MYASTHENIA GRAVIS: HUMORAL FACTORS

Regulation of B-Lymphocyte Activation, Proliferation, and Differentiation

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Understanding the regulation of antibody synthesis is central to any effort to control the disordered immune responses which underlie many autoimmune disorders such as myasthenia gravis. I will discuss here the mechanisms involved in the stimulation of B lymphocytes, the cells which are the precursors of antibody-secreting cells. Emphasis will be placed on the activation of B cells from their resting state, the regulation of their proliferation, and some features of their differentiation into antibody-secreting cells, including a brief consideration of the problem of class switching.

B lymphocytes are derived from precursors in hematopoietic tissue. Much has recently been learned about the biochemical and genetic events that control the assemblage of immunoglobulin (Ig) genes in B-cell precursors.[1] From this information, a picture is emerging of the mechanisms through which antibody diversity is achieved. Much less is known about the regulation of pre-B-cell growth and development. An important recent contribution to this field has been the introduction of a system through which pre-B cells may be cultured on monolayers of bone marrow stromal cells.[2] In such cultures, pre-B cells proliferate and undergo some differentiation into mature B cells. The stromal cell monolayer appears to be critical to these processes, presumably reflecting the production of growth-regulatory substances by stromal cells. Efforts to characterize the stromal cells and to identify and purify the growth factors are now in progress. Work of this type promises to yield information of major significance to our ability to regulate the overall level of B-cell production.

TWO MECHANISMS OF B-CELL ACTIVATION

Pre-B cells differentiate into membrane Ig-bearing B cells, which initially exist in a resting, or G_o, state. Antibody responses to substances introduced into an individual for the first time depend upon the existence in the individual of B cells that bear

membrane receptors complementary to antigenic determinants (epitopes) on the foreign molecule and on the activation and expansion of such resting cells.

In recent years, it has become clear that two apparently distinct mechanisms have evolved through which resting B cells may be specifically activated.[3] One of these, often referred to as receptor cross-linkage stimulated B-cell activation, depends upon the binding of antigen to the membrane receptor of the B cell and the subsequent generation within the B cell of a series of biochemical signals, which I will describe below. Signal generation in such cases requires that receptor molecules be cross-linked to one another by binding to multiple epitopes on the same backbone. Since all of the receptors on an individual B cell have the same binding site, antigens capable of activating B cells by receptor cross-linkage must possess multiple copies of the same epitope (or must be rendered multivalent by some mechanism). There are important classes of molecules that bear multiple copies of the same epitope. These include polysaccharides, such as the constituents of the capsules of pyogenic microorganisms, and polynucleotides. Antigens with such repetitive epitopes have been designated type-2 antigens.[4] Antibody responses to type-2 antigens were formerly believed to be independent of the need for help from T lymphocytes. It is now clear that antibody responses to type-2 antigens do require T-cell products,[5] such as B-cell stimulatory factor-1 (BSF-1), B-cell growth factor-II (BCGF-II) and interleukin-2 (IL-2). However, the initial activation of B cells by type-2 antigens does not require the active participation of T cells, and thus these responses are relatively less dependent on T cells than are responses to the so-called thymus (T)-dependent antigens. The T-dependent antigens are typically proteins, particularly those that bear no more than one copy of any individual epitope. (Proteins express multiple distinct epitopes, but generally each exists only once in a given molecule unless the protein contains multiple identical polypeptide chains.) T-dependent antigens are unable to cross-link membrane receptors of B cells and thus do not directly create biochemical signals within the B cell to which they bind. The activation of such cells depends upon an intimate interaction with a T lymphocyte that is, itself, specific for an epitope on the same antigen that was bound by the B cell. This activation pathway depends upon cognate, T-cell-B-cell, interaction and involves the co-recognition by the T cell of an epitope and a class II major histocompatibility complex (MHC) molecule on the surface of the B cell.

Receptor Cross-Linkage Stimulated B-Cell Activation

Cross-linkage of membrane immunoglobulin on the surface of B cells can most conveniently be achieved by anti-Ig antibodies. Normal resting B cells and Ig-bearing B lymphoma cells demonstrate a rapid increase in inositol phospholipid metabolism in response to anti-Ig antibodies.[6-9] In cells in which the inositol phospholipid pool has been labeled with tritium, there is a striking increase in radioactivity in inositol trisphosphate (IP3) within 15-30 seconds. The elevation of IP3 reflects hydrolysis of phosphatidyl inositol bisphosphate (PIP2) by phospholipase C (PLC). It appears most likely that receptor cross-linkage leads to enhanced PLC activity, initiating these events. Thus far, no molecular intermediates between membrane Ig and PLC have been identified, and the initial events in signal transduction are unknown.

The cleavage of PIP2 yields IP3 and diacylglycerol (DG). Both of these products have biological activities which appear to be of critical importance to the activation of the B cell. IP3 mobilizes calcium from intracellular stores and thus causes an

elevation in intracellular free calcium concentration ($[Ca^{++}]_i$).[10,11] DG causes the activation of protein kinase C (PKC),[12,13] an enzyme which catalyzes the phosphorylation of several proteins of B cells, most notably plasma-membrane-associated proteins and certain cytoskeletal proteins.[14] The importance of increased $[Ca^{++}]_i$ and of enhanced PKC activity in B-cell activation is strongly suggested by the observation that B cells can be stimulated by a combination of agents which increase $[Ca^{++}]_i$ and enhance PKC activity. These are the calcium ionophores, such as A23187 and ionomycin, and phorbol esters, such as phorbol myristate acetate (PMA). Indeed, treatment of B cells with a calcium ionophore and PMA leads to responses which are very similar to those observed in response to anti-IgM antibodies, including entry into the G_1 phase of the cell cycle, induction of class II MHC molecule expression, and sensitivity to the growth-stimulating activity of BSF-1.[15,16] Thus, receptor cross-linkage leads to B-cell activation mediated largely through the inositol phospholipid metabolic pathway and involving elevation in $[Ca^{++}]_i$ with the attendant increase in activity of a myriad of calcium-dependent enzymes; and activation of PKC, leading to the phosphorylation (and presumed change of function) of several B-cell proteins.

A second important event in the activation of the resting, or the early G_1, B cell is the binding of BSF-1 to that cell. BSF-1 is a 20,000 M_r T-cell product that has been purified to homogeneity[17-19] and for which a monoclonal antibody[20] and a cDNA clone have recently been obtained. This lymphokine acts on resting B cells, as shown by the facts that it causes a substantial increase (~6-fold) in the amount of class II MHC molecules expressed on B cells[21,22]; and it prepares cells to respond more promptly to anti-Ig antibodies.[23,24] Resting B cells have recently been shown to express small numbers (~300) of high-affinity receptors for BSF-1.[25]

Although the biochemical basis of BSF-1 action has not yet been established, it plays a critical role in the entry into the S phase of the cell cycle of B cells that have been treated with anti-Ig antibodies.[26] Thus, resting B cells stimulated with low concentrations (1–5 μg/ml) of anti-Ig alone enter G_1 but not S phase, unless they also receive BSF-1 as resting- or early-G_1-phase cells. Anti-Ig and BSF-1 may be considered to act jointly to induce a state of competence in B lymphocytes for cellular proliferation.[27]

The mechanism(s) through which such competent B cells are stimulated to proliferate is not clear. Several possibilities exist. There may be a unique B-cell growth factor that acts as a late G_1 "progression" factor and that stimulates cell division by B cells prepared through the joint action of anti-IgM and BSF-1. There is emerging evidence that the factor designated BCGF-II,[28] for which a cDNA clone has recently been obtained,[29] displays this function. Indeed, some human B lymphoma cells and normal activated human B cells produce a B-cell growth factor that may act as an autocrine growth stimulant.[30,31] Thus, it is possible that induction of a state of competence in B cells renders them sensitive to the action of a B-cell growth factor that is supplied to them either as a T-cell product or as an autocrine (B-cell-derived) growth stimulant. A second possibility is that BSF-1 acts not only as a co-competence factor but also as a late-G_1 progression factor. BSF-1 in high concentrations has some growth-stimulatory activity on mouse B-cell blasts,[27] and BSF-1 has also been recently shown to stimulate growth of certain T-cell lines.[32] A third possibility is that interleukin-2 (IL-2), which has been shown to act on some IL-2 receptor-bearing B-cell blasts[33] and B lymphoma cells,[34] is a progression factor for many competent B cells. Finally, B cells that have been activated with anti-Ig and BSF-1 or with a high concentration of anti-Ig alone may enter S phase without the need for any progression factor. Recent progress in the purification of B-cell active proteins such as BSF-1, BCGF-II, and IL-2 and in the identification and purification of the receptors for these growth stimulants[35,36] makes it likely that the control of B-cell proliferation in the

receptor cross-linkage stimulated B-cell activation pathway will be understood in more precise terms in the near future.

Ig synthesis by activated, proliferating B cells is also controlled by the action of lymphokines. Human B-lymphocyte Ig secretion has been shown to be stimulated by a factor designated B-cell differentiation factor (BCDF or B-cell stimulatory factor-2 [BSF-2]).[36] In the mouse, BSF-2 has not yet been identified. B-cell differentiation is stimulated by several agents, including BCGF II (or a related molecule) in synergy with IL-2.[37]

Cognate T-Cell-B-Cell Interactions

Although our understanding of receptor cross-linkage stimulated B-cell activation is far from complete, substantial progress has been made in purifying the growth-regulatory polypeptides and in determining the cellular biochemical events that govern this response. Unfortunately, much less information has been obtained concerning the form of B-cell activation mediated by cognate T-cell-B-cell interaction. This is, at least in part, due to the difficulty of studying systems involving the action of two distinct cell types.

Nonetheless, certain important insights have been gained regarding this form of B-cell activation. It seems clear that a critical element in cognate T-cell-B-cell interaction is the capacity of B cells to process and present antigen to T cells. Thus, antigens are bound by B-cell receptors based on their structural complementarity. In general, B cells recognize epitopes found on intact, native antigens, while epitopes recognized by T cells consist of a complex composed of a peptide cleaved from the intact antigen and a class II MHC molecule.[38] For cognate T-cell-B-cell interactions to be achieved, the native antigen bound to the B cell receptor must be endocytosed and then must undergo proteolytic degradation.[39] Some of the peptides formed as a result of proteolysis are reexpressed on the B-cell membrane. Recent evidence indicates that these peptides form noncovalent complexes with class II MHC molecules,[40] which are then recognized by the T cell. This recognition may serve to bring already-activated, specific T cells into contact with specific B cells; or it may lead to the activation of a resting T cell, causing it to secrete lymphokines and possibly to divide. On the last point, there is still uncertainty as to whether a resting B cell expressing an antigen-MHC complex on its surface is capable, by itself, of stimulating a resting T cell to secrete lymphokines.

The molecular mechanism of B-cell activation in cognate T-cell-B-cell interaction is not clear; several alternative ideas have been proposed. The interaction of the T cell with antigen-MHC molecules on the B-cell surface might generate a biochemical signal within the B cell, presumably mediated by the class II MHC molecule. The question of whether class II molecules can transmit signals has not been fully resolved; it is thus premature to judge the likelihood that an activation signal is directly generated by cellular coupling in cognate interactions. A second possibility is that in the microenvironment of the T-cell-B-cell interaction, the T cell produces lymphokines, which signal the B cell to activate and to divide. In favor of this idea are the finding that stimulated T cells produce lymphokines and recent data that suggest that the secretory apparatus of T cells involved in T-cell-B-cell interactions is polarized to the region of the interaction.[41] This suggests that stimulatory lymphokines may be directionally secreted to act principally upon the B cell to which the T cell is bound. However, the best characterized T-cell-derived lymphokines lack the capacity by

themselves to prepare a resting B cell to divide. BSF-1 requires a co-stimulant, such as anti-IgM, to render a resting B cell competent to divide in response to a progression factor. It is possible that the T cell produces a second lymphokine that acts with BSF-1; or that it produces a lymphokine that, by itself, induces competence, or even causes the resting B cell to enter S phase without any other stimulant.[42] Yet another possibility is that locally produced BSF-1 synergizes with a postulated signal created in the B cell by the direct interaction of the B cell with the T cell, or through the action of an antigen-specific T-cell product that binds to the B-cell surface.

Although these issues are far from being resolved, it is already clear that the interplay of T-cell-derived lymphokines can have very important effects on B cells and that major regulatory events mediated by lymphokines occur in the context of T-cell–B-cell interactions. One example is the antagonism of BSF-1 function by interferon γ (IFNγ). It has recently been shown that many of the actions of BSF-1 on resting B cells are inhibited by IFNγ. These actions include the preparation of the cells to respond more promptly to progression factors[43] and the induction of expression class II molecules on B cell surfaces.[44] Mosmann and colleagues[45] have recently reported that many T-cell clones produce IL-2 and IFNγ, but not BSF-1 (Th1 cells); while other T-cell clones produce BSF-1, but not IL-2 or IFNγ (Th2 cells). This would suggest that the stimulation of a B cell by BSF-1 in a cognate interaction with a Th2 cell might be inhibited by a cognate interaction of a Th1 with the same B cell. Indeed, this would lead to the possibility that Th1 cells, which under most circumstances would be regarded as helper/inducer cells, may *suppress* responses of resting B cells that are dependent upon the action of BSF-1.

Once a B cell is activated as a result of a cognate interaction with a specific T cell, it is likely that its requirements for entry into S phase and for differentiation into an antibody-secreting cell are similar to those of B cells activated through receptor cross-linkage.

BSF-1 CONTROLS SWITCHING OF IG-CLASS EXPRESSION

One of the most important and poorly understood aspects of antibody responses is the phenomenon of Ig-class switching. Its importance lies in the distinctive biological function of the various Ig classes. It has recently been demonstrated that class switching may be controlled by soluble factors.[46] The best example of this is the effect of BSF-1 on the production of IgG1[47] and IgE[48] by B cells stimulated with lipopolysaccharide (LPS). When resting B cells are stimulated with LPS, they secrete IgM and, to a lesser degree, IgG3. Addition of purified BSF-1 to the cultures with LPS leads to the appearance of significant amounts of IgG1 and IgE, and to a diminution in the amount of IgG3 made. This effect appears to involve Ig-class switching, since the precursors of the IgG1-producing cells lack membrane IgG1 when they are initially stimulated with BSF-1.[46] BSF-1 plays an important role in the control of Ig-class expression *in vivo* as well as *in vitro*. Injection of larvae of the helminth *Nippostrangylus brasiliensis* causes a 100-fold increase in serum IgE levels. If mice are treated with a monoclonal antibody to BSF-1 at the same time that the larvae are injected, the increase in serum IgE concentration is inhibited by 10-fold.[49] This is very strong evidence that endogenous production of BSF-1 has a critical effect in switching to the expression of IgE in mice with parasitic infections. The molecular mechanisms underlying Ig-class switching in response to BSF-1 are now under investigation.

CONCLUSIONS

The activation of resting B cells to enter G_1 phase, to undergo clonal expansion through proliferation, to differentiate into Ig-secreting cells, and to switch expression of Ig class are highly regulated events. As described in this paper, B cells have at least two mechanisms for their initial stimulation; but thereafter it seems likely that their control mechanisms will prove to be rather similar. Substantial progress has been made in many aspects of this work as a result of the chemical purification of the growth-regulatory factors, the preparation of monoclonal antibodies to these factors, and the derivation of DNA clones—all of which makes possible detailed analysis of regulation of factor production and allows the production of virtually unlimited amounts of these enormously potent signalling molecules. Among the key areas for future study will be the development of a quantitative physiology for lymphocyte responses. Progress in this area should prove very useful in elucidating the role of disordered immune regulation in autoimmune diseases.

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(x) Related Proceedings Appendix

In support of section (ii) regarding Related Appeals and Interferences, Applicants submit and attach hereto a copy of the Non-Final Office Action issued on February 20, 2008, in which the then pending rejections of claims 21-23 and 32-34 were withdrawn.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/734,024	12/11/2003	David B. Weiner	UPVG0005-101	2356

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EXAMINER	
HUMPHREY, LOUISE WANG ZHIYING	
ART UNIT	**PAPER NUMBER**
1648	
MAIL DATE	**DELIVERY MODE**
02/20/2008	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	10/734,024	WEINER ET AL.	
	Examiner	Art Unit	
	LOUISE HUMPHREY	1648	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *30 November 2007*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *21-23 and 32-34* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *21-23 and 32-34* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

Response to Amendment

In view of the brief filed on November 30, 2007, PROSECUTION IS HEREBY REOPENED. See the rejections set forth below.

To avoid abandonment of the application, appellant must exercise one of the following two options:

(1) file a reply under 37 CFR 1.111 (if this Office action is non-final) or a reply under 37 CFR 1.113 (if this Office action is final); or,

(2) initiate a new appeal by filing a notice of appeal under 37 CFR 41.31 followed by an appeal brief under 37 CFR 41.37. The previously paid notice of appeal fee and appeal brief fee can be applied to the new appeal. If, however, the appeal fees set forth in 37 CFR 41.20 have been increased since they were previously paid, then appellant must pay the difference between the increased fees and the amount previously paid.

A Supervisory Patent Examiner (SPE) has approved of reopening prosecution by signing below:

/Bruce Campell/
Supervisory Patent Examiner, Art Unit 1648.

Claims 1-20 and 24-31 have been cancelled. Claims 21-23 and 32-34 are pending and currently examined.

Claim Objections

Claim 21 is objected to because of the following informalities: the claim refers to the proteins Vpr by its acronym without first identifying it by the full name, HIV-1 viral protein R. Appropriate correction is required.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Applicant's arguments, see pages 11-16 and 20-24, filed on 30 November 2007 in the appeal brief, with respect to the rejection of claims 21-23 and 32-34 under 35 U.S.C. §103(a) as being unpatentable over Rogel *et al.* (February, 1995) have been fully considered and are persuasive. Therefore, the rejection has been withdrawn. However, upon further consideration, a new ground of rejection is made in view of the different interpretation of the previously applied reference. See the new enablement rejection below.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

The rejection of claims 1-5, 14, 15, 20, 25-27 and 29-34 under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification commensurate in scope is **withdrawn** in response to Applicants' amendment.

New Rejection: Claims 21-23 and 32-34 are rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement. The claims contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

In making a determination as to whether an application has met the requirements for enablement under 35 U.S.C. 112 ¶ 1, the courts have put forth a series of factors (MPEP §2164.01(a)). See, *In re Wands*, 8 USPQ2d 1400, at 1404 (CAFC 1988); and *Ex Parte Forman*, 230 U.S.P.Q. 546 (BPAI 1986). The factors that may be considered include (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims. *Id.* While it is not essential that every factor be examined in detail, those factors deemed most relevant should be considered.

The nature of the invention is the inhibition and prevention of lymphocyte activation by Vpr protein. The breadth of the claims encompasses both B lymphocytes and T lymphocytes and both *in vitro* and *in vivo* prevention and inhibition.

The disclosure fails to provide any working embodiments that meet the claimed limitations. While there are examples of assays to identify Rip-1-binding fragments of Vpr that are inducers or inhibitors of glucocorticoid receptor (GR) type II complex

translocation from cytoplasm to the nucleus, no *in vitro* or *in vivo* working example of any prevention or inhibition of lymphocyte activation is disclosed in the specification.

The specification provides no guidance regarding practice of the claimed methods. The specification refers generally to the Vpr's interaction with the glucocorticoid steroid biochemical pathway (page 22, line 26-37), that the expression of Vpr within the cell causes the cell to stop proliferating (page 5, line 31-35) and that Vpr inhibits cytokine production/secretion by T cells, B cells, and monocytes during immunoglobulin activation (page 10, lines 14-20). However, the disclosure is silent pertaining to specific method steps of inhibition and prevention of lymphocyte activation. The disclosure fails to provide any guidance pertaining to the structural characteristics or mechanisms of the interaction between Vpr and lymphocytes. The specification specifically discloses in more details and in working examples the use of Vpr or Rip-1-binding fragments of Vpr protein as transfection agent for the delivery of conjugated nucleic acid molecule or derivatives into the nucleus of a cell (page 36-37), which is not remotely related to inhibition or prevention of lymphocyte activation. Therefore, the disclosure does not correlate with the claimed method of preventing and inhibiting lymphocyte activation *in vitro* or *in vivo*, especially inside humans.

At the time the invention was made, successful implementation of lymphocyte activation inhibition and prevention with Vpr was not routinely practiced by those skilled in the art. Prior art only teaches T lymphocytes to secrete cytokines upon activation (Mosmann, 1997) and B lymphocytes to produce immunoglobulins once activated by cytokines (Paul, 1987). The only effect of Vpr expression within cells is the alteration of

distribution of cells in the cell cycle and thereby mediating the prevention of cell proliferation (Rogel, February 1995). The prior art is unpredictable and fails to provide sufficient illumination pertaining to the mechanisms underlying inhibition and prevention of lymphocyte activation by the Vpr protein.

There is no specific guidance in the art or specification and no specific examples of the claimed method set forth in the specification. While Applicant is not required to set forth working examples, the specification must set forth sufficient teachings to allow one to practice the claimed invention. Legal precedence dictates that the scope of the claims must bear a reasonable correlation to the scope of enablement provided by the specification. *In re Fisher*, 427 F.2d 833, 839, 166 U.S.P.Q. 18 24 (C.C.P.A. 1970). *In re Vaeck*, 20 U.S.P.Q.2d 1438 (C.A.F.C 1991). *In re Angstadt*, 537 F.2d 498, 502-03, 190 U.S.P.Q. 214, 21 (C.C.P.A. 1976). There is no evidence that Vpr has any effect on T lymphocyte secretion of cytokines, let alone the any effect on the activation of B lymphocytes. Thus, when all the aforementioned factors are considered *in toto*, it would clearly require undue and unpredictable experimentation from the skilled artisan to practice the claimed invention.

In conclusion, the instant invention, based on the evidence as a whole, in light of the factors articulated by the court in *In re Wands*, lacks an enabling disclosure.

Correspondence

Any inquiry concerning this communication or earlier communications from the examiner should be directed to LOUISE HUMPHREY whose telephone number is

(571)272-5543. The examiner can normally be reached on Mon-Thu, 9:00 am - 5:30 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Bruce Campell can be reached on 571-272-0974. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/L. H./
Examiner, Art Unit 1648

/Bruce Campell/
Supervisory Patent Examiner, Art Unit 1648